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THE FLUORESCENCE OF 3 : 4-BENZPYRENE *IN VIVO*. PART I: THE DISTRIBUTION OF FLUORESCENCE AT VARIOUS SITES, ESPECIALLY THE SKIN OF MICE.*

I. DONIACH, J. C. MOTTRAM AND F. WEIGERT.

From the Physico-Chemical Department of the Mount Vernon Hospital, Northwood, Middlesex, England.

Received for publication December 15, 1942.

SHORTLY after painting the skin of mice with 3 : 4-benzpyrene, either in solution in acetone or benzene or in watery suspension, it fluoresces brilliantly violet. We have shown that this violet fluorescence changes within a few days to blue (Mottram and Weigert, 1942 ; Weigert, 1941 ; Weigert and Mottram, 1940).

Fluorescence spectrography shows that the violet fluorescent light has a main band at 427 mμ. and weaker diffuse bands at 405 and 455 mμ. (Fig. 1, *a*). Its spectral structure is identical with that of the fluorescence of molecularly dispersed solutions of benzpyrene. The spectrum of the blue-fluorescent skin is quite different, with a main diffuse band at 450 mμ. and a weaker diffuse band at 425 mμ. (Fig. 1, *b*). This "blue" spectrum is similar to, but not identical with, the spectrum of "BPX" (Fig. 1, *c*) which Peacock (1936) and Chalmers (1938) found secreted with the bile of animals treated with benzpyrene, and also resembles the spectrum of the orthorhombic metastable crystals of benzpyrene (Weigert and Mottram, 1940) (Fig. 1, *d*). The skin of untreated mice shows a whitish-blue fluorescence spectrum having a broad band extending from 420 to 500 mμ. (Fig. 1, *g*).

The gradual change in the fluorescence spectrum of the skin of white mice from violet to blue over a period of four days after painting with benzpyrene is shown in Fig. 2. This change has been observed in many cases.

A. *Technique for Producing Fluorescence Spectrograms of Biological Specimens.*

A 125-watt G.E.C. Osira mercury arc lamp (Fig. 3) with a Wood glass bulb and an additional Wood glass filter, F, was used as a light source for the fluorescence experiments. The quartz burner, Q, was focused as a narrow line of 4 mm. length by means of a large condenser, C, of ordinary glass upon the portion of the skin or other specimen under test. The fluorescent light which emerged from that line passed again through the condenser, and the central portion of the cone was deflected by a glass prism, P, on to the slit, S, of a high-aperture glass spectrograph by Adam Hilger, Ltd., of London (see Fig. 3). This autocollimating arrangement guaranteed the recording of the most important portions of the fluorescent light, which emerge from the surface layers of

* This investigation was aided by a grant from the British Empire Cancer Campaign.

the specimen, and troubles arising from absorption of the fluorescent light by the specimen itself were greatly reduced. With a spectrograph slit of 0·4 mm. width and supersensitive panchromatic Kodak "P 800" plates, exposures which varied from a few seconds to 10 minutes, according to the intensity of the fluorescence, secured good spectrograms. The fluorescence was chiefly excited by the intense lines of the mercury arc near 365 mμ. They did not reach the photographic plate, but the violet line at 405 mμ. was transmitted by the Wood glass, and served as a reference mark for a wave-length scale in the spectrograms. Fluorescence spectograms produced with other illumination devices, spectrographs or brands of photographic material may be different from those shown in Figs. 1 and 2 : for instance, with a quartz spectrograph the main band of benzpyrene in acetone solution would be at 405 mμ. and not at 427 mμ. as in our Fig. 1, *a*.

B. *Experiments in which Mice were Painted Repeatedly and Kept Alive for Several Months.*

Three groups of 12 mice each were painted twice a week with (*1*) 0·5 per cent. benzpyrene in benzene, (*2*) 0·5 per cent. benzpyrene + 0·01 per cent. naphthacene in benzene, and (*3*) 0·01 per cent. naphthacene in benzene. The skin of one mouse from each group, after clipping the hair, was spectrographed daily, so that each mouse was exposed to ultraviolet radiation only about once in 12 days.

The naphthacene was introduced in group (*2*) as an experimental convenience for the following reason : The blue fluorescence emitted by pure orthorhombic crystals of benzpyrene is quenched by minute amounts of naphthacene in solid solution, and the very much stronger green fluorescence of naphthacene is seen instead (Weigert, 1940). This provides a "fluorescence label" for small amounts of orthorhombic crystals of benzpyrene. This is illustrated in Fig. 1, *d*, *e*, which shows the fluorescence spectrograms of suspensions of the orthorhombic form of benzpyrene without (1, *d*) and with (1, *e*) naphthacene ; the fluorescence label in (1, *e*) is very clear. Group (*3*) served as a control. The following results were obtained :

(*a*) In groups (*1*) and (*2*) the violet fluorescence was gradually replaced by the blue during the first four days after painting, as seen in Fig. 2.

(*b*) In group (*2*) green naphthacene bands were seen a few hours after painting, indicating that benzpyrene had crystallized on the surface of the skin in the orthorhombic form. The green bands disappeared after one day, showing that the benzpyrene had entered the skin as a true solution. The continued absence of the green

---

FIG. 1.—Fluorescence spectrograms of : (*a*) *Benzpyrene in acetone solution*, 1/400,000. (*b*) *Blue-fluorescent skin*, one day after painting with benzpyrene in acetone, excess benzpyrene removed with benzene. (*c*) *Blue-fluorescent mouse bile*, 6 hours after intravenous injection of benzpyrene suspension ; diluted with water. (*d*) *Suspension of the blue-fluorescent orthorhombic form of benzpyrene*, 1/50,000 in water. (*e*) *Same as* (*d*) *with* 1 *per cent. naphthacene* in solid solution, fluorescence green. (*f*) *Sky-blue fluorescent areas on rabbit lung* after injection of benzpyrene. (*g*) *Bluish-white fluorescence of normal mouse skin*. The maxima of the fluorescence, which can be better seen on the original negative spectrograms, are marked by white dots.

FIG. 2.—Spectrograms of the fluorescence of the skin of 10 *living* mice from one hour to four days after painting with benzpyrene in benzene. Hair clipped. Excess benzpyrene not removed. The band of the blue form at 450 mμ. develops slowly from the band of the violet form at 455 mμ.

FIG. 3.—Arrangement for spectrography of the fluorescence of biological specimens. Q, Quartz mercury burner ; C, glass condenser ; F, filter ; P, glass deflection prism ; S, slit of spectrograph (in adjacent room).

FIG. 4.—Photomicrographs (× 5) of the fluorescence of the detached transparent skins of white mice under xylol : (*a*) 1 hour, (*b*) 24 hours after painting the skins with benzpyrene in acetone. Excess benzpyrene removed with benzene. The pattern of the rows of bulbs fluoresces blue in (*b*), but not yet in (*a*).

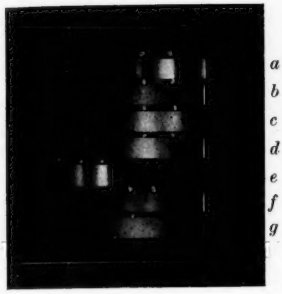


FIG. 1.

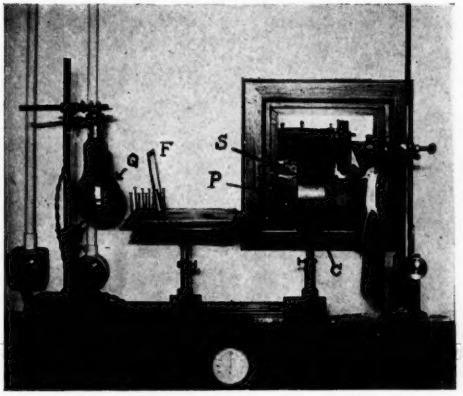


FIG. 3.

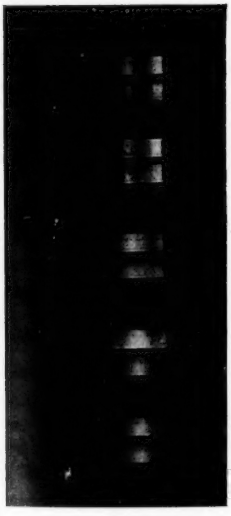
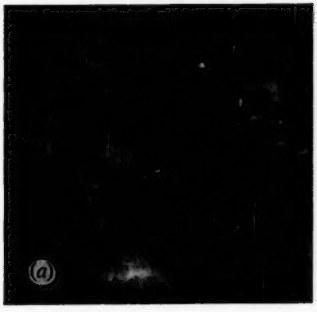


FIG. 2.



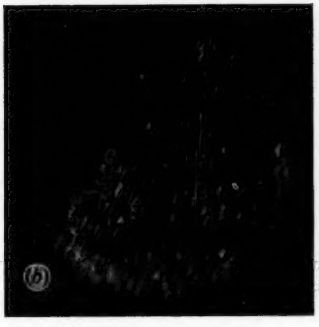


FIG. 4.

Doniach, Mottram and Weigert.

bands throughout the later stages of the experiment provided evidence in support of the contention that the blue-fluorescent compound, formed in the skin, is not identical with the orthorhombic form of benzpyrene (see Discussion).

(*c*) In group (*3*) only the natural fluorescence of the skin was seen, and at no stage did the green bands of naphthacene appear, because no foreign crystals which could act as sensitizers for the fluorescence were present in the skin in this control group.

The observations were continued until tumours occurred in about three months; they appeared in groups (*1*) and (*2*), but not in group (*3*).

c. *Experiments in which the Painted Skins were Spectrographed after Removal.*

Mice were painted once with 0·5 per cent. benzpyrene in benzene and killed after 1–34 days. The painted skin of the back was clipped and then removed and fat and connective tissue were dissected away and examined separately. Spectrograms were taken of the skin from the fur side and from the inner side, and of the separated connective tissue, and the relative intensities of the violet (v), the blue (**b**) and the natural (n) fluorescences were approximately estimated from the densities of the various bands in the spectrogram on a scale from $\frac{1}{4}$ to 5. The results are presented in Table I, the blue fluorescence of particular interest being shown in bold type.

TABLE I.—*Change of Relative Violet* (v), *Blue* (**b**) *and Natural* (n) *Fluorescence Intensities with Time.*

Days . .	1.	2.	3.	4.	5.	6.	7.	10.	12.	13.	16.	21.	34.
Skin (fur) . .	5 v	5 v	v, **b**	v, **b**	v, **2b**	v, **2b**	v, **b**	v, **2b**	**b**, $\frac{n}{2}$	$\frac{v}{2}$, **2b**	$\frac{v}{2}$, **b**	$\frac{\mathbf{b}}{4}$, n	n
Skin (inside) .	4 v	4 v	v, **2b**	v, **2b**	$\frac{v}{2}$, **2b**	v, **3b**	v, **b**	$\frac{\mathbf{b}}{2}$, n	$\frac{\mathbf{b}}{2}$, n	$\frac{\mathbf{b}}{2}$, n	$\frac{\mathbf{b}}{2}$, n	n	n
Connective tissue	v, n	$\frac{v}{2}$n,	n	n	$\frac{v}{2}$, n	n	n	n	—	—	—	—	—

Muscular tissue under the skin showed only natural fluorescence, except in rare cases where contamination was obvious. The table shows that both the violet and the blue fluorescence persisted with gradual diminution for about 2–3 weeks. The blue fluorescence was first seen on the third day and on the inside of the skin persisted until the 16th day, whereas the violet had disappeared after the 7th day. The violet fluorescence persisted on the fur side for 2–3 weeks, and this was probably due to its continued presence upon or within the hair, which could not be entirely removed by clipping.

D. *Fluorescence Phenomena in Mice and Rabbits after Intravenous Inoculation of Benzpyrene.*

Mice (tail vein) and rabbits (ear vein) were inoculated with saline suspensions of benzpyrene, 0·3–0·5 mg. into mice and 6–15 mg. into rabbits.

We confirmed Peacock's (1940) findings that the bile fluoresced blue ("BPX") with maxima at about 425 and 450 mμ. within a few minutes after inoculation, and Chalmers's (1938) evidence that the faeces fluoresced greenish-blue ("BPF") with maxima near 440 and 460 mμ. when collected one day after the inoculation. Adipose tissue fluoresced violet about 30 minutes after the inoculation (Peacock, 1940).

Blue fluorescence with spectral maxima at about 450 and 425 mμ. was also observed

at four other sites (Mottram and Weigert, 1942 ; Weigert, 1941 ; Weigert and Mottram, 1941).

(1) The *kidneys* of mice and rabbits showed violet fluorescence shortly after the inoculation, changing in the course of 1–2 hours to a blue fluorescence. After 4 hours no violet remained with mice. The blue persisted for more than 24 hours, and had disappeared after 2 days ; it was confined to the cortical region. Two to four hours after injection the cortical region of rabbit kidneys fluoresced blue, the medullary parts showed only natural fluorescence, while the pelvis showed traces of violet ; the blue fluorescence showed the same bands as the blue found in the skin.

(2) The *lungs* of mice fluoresced bright yellow for about 10 minutes after the injection, due to trapped particles of the suspension injected, and afterwards violet fluorescence slowly supervened. This agrees with Peacock's (1940) statements. In addition, we observed after 2–4 hours traces of blue fluorescence similar to the blue of the skin. One day after inoculation only natural fluorescence was seen.

In rabbits blue fluorescence was seldom clearly seen ; instead, blue-green fluorescent patches of remarkable brilliance, having bands at about 440 and 460 mμ., sometimes covered large areas of the surface and sometimes small (see Fig. 1, *f*). Although 26 rabbits were used, the conditions governing this phenomenon were not discovered ; sometimes it was clearly seen, while at others it was entirely absent.

The blue-green fluorescence was more easily removed by ether from the lungs than was the blue from the kidney cortex. On shaking the ether extract with alkali a yellow-green fluorescence entered the aqueous phase, from which the blue-green could be again extracted with ether after acidification. These reactions are like those described by Chalmers (1938) for " BPF " of the faeces which Chalmers and Crowfoot (1941) identified as a monohydroxy-benzpyrene.*

(3) The *livers* of mice showed weak violet fluorescence 2–4 hours after injection, and subsequently the typical bands of the blue slowly appeared in the spectrum. After one day the liver showed only the natural fluorescence. In rabbits the blue fluorescence was masked by the strong yellow-green natural fluorescence of the normal liver.

(4) The *mammary glands* of mice showed violet fluorescence as described by Peacock (1940). The blue fluorescence was not seen, but was found in the milk taken from the stomachs of suckling mice when the mother was inoculated 3–4 days after littering. When the young were killed 4–6 hours after inoculation of the mother and the milk extracted twice with ether to remove the violet fluorescence in the fat, the aqueous remainder which contained the proteins fluoresced blue, and showed the typical bands which appeared in the skin. This was not found earlier than 4 hours after inoculation.

In no other organ except the intestine was blue fluorescence found ; this is being dealt with in a following paper.

E. *Microscopical Examination of the Mouse Skin and Paramecium.*

White mice were painted with 0·3 per cent. benzpyrene in acetone upon the abdomen after clipping the hair. They were killed from a few hours to 8 days afterwards. The

* Still another blue fluorescence was seen in the lungs of mice due to a post-mortem process. If the mice were killed shortly after inoculation the bulk of the yellow-fluorescent benzpyrene particles was still trapped in the lung capillaries. The lungs were stored in ice for a few days. If the interval between inoculation and killing was no more than one minute, this yellow fluorescence changed to a bright blue, which increased in intensity slowly. Its spectrum had only one maximum, at about 460 mμ., and it simulated the spectrum of a strongly enhanced natural fluorescence of the proteins (see Fig. 1, *g*). If the mouse was killed later than one minute after the injection this aberrant blue spectrum was slowly replaced by the violet. Study of the phenomenon is still in progress.

skins were shaved and removed, fat and connective tissue were dissected away, and the skins were then dried and mounted between glass slides. They were examined microscopically, using a carbon arc lamp screened by Wood glass and copper sulphate solution.

One to six hours after painting the skins showed only a general fluorescence, with a smudgy brightness corresponding to the position of the sebaceous glands ; these are well seen in visible light as black twin sacs after treating the skins with benzene and then drying them. After one to two days the general violet fluorescence was much less pronounced, whereas the hair-bulbs shone sharply and brilliantly blue. After three or four days only the rows of hair-bulbs were visible, the fluorescence of the sebaceous glands having entirely disappeared. Subsequently the blue fluorescence faded, and it was not visible at all after the 6th day, although found at much later stages spectroscopically (see Table I). These phenomena are illustrated in Fig. 4 by photomicrographs ($\times$ 5) of the fluorescence of the detached transparent skin under xylol : (*a*) 1 hour, (*b*) 24 hours after painting with benzpyrene. In (*b*), but not yet in (*a*), the rows of bulbs show strong blue fluorescence.

Paramecium, Pleuronema and other Ciliates were placed in a 1/100,000 watery suspension of benzpyrene. After varying times some were removed, fixed with formalin vapour, and at once examined microscopically in ultraviolet light. Within a few minutes the food vacuoles were seen to be filled with yellow-fluorescent colloidal benzpyrene, and within about half an hour the cytoplasm showed an even violet fluorescence. The macro-nucleus did not fluoresce, and so was represented by a dark area. After four hours the violet fluorescence showed a granular structure, especially close to the surface and at the two ends in the case of Paramecium. The intensity of the fluorescent granules increased and the colour gradually changed to a bluish-white, so that by the 3rd to 4th day Paramecium had the following remarkable appearance in ultraviolet light. The cytoplasm, containing food vacuoles fluorescing yellow, fluoresced violet except where the nucleus made a dark area ; it also showed granular structure with bluish-white fluorescence forming a band at the surface of the organism which was thickest at the two ends. There was also some blue " granular " fluorescence near the centre around the nucleus. Spectrograms of Paramecium in this state which had been spun down in a glass capillary showed chiefly the violet spectrum, with slight indications of the typical blue band at 450 mμ. The blue fluorescence observed with the naked eye is not reliable.

These observations confirm Graffi's (1939) findings on Paramecium with benzpyrene. He established that the nucleus did not fluoresce. He also observed " granular " fluorescence distributed over the entire body, and came to the conclusion that the fluorescing granules were mitochondria.

Thus both in the hair-bulbs of the skin and in Paramecium are seen microscopically gradual changes from violet to blue fluorescence, similar to the change of fluorescence observed macroscopically and by spectrographic analysis.

F. *Amount of Benzpyrene left in the Skin of Mice after Painting.*

Mice were clipped on both flanks and on the abdomen. They were then painted on each area with 0·03 ml. of 0·3 per cent. benzpyrene in acetone, or 0·27 mg. of benzpyrene in all. The mice were killed after $\frac{1}{4}$, 1, 2, 6, 24 and 48 hours. In the $\frac{1}{4}$-hour and 1-hour experiments the mice were loosely fixed to cork in order to avoid loss by movement and rubbing. After killing, the painted areas of the skins were removed, fat and connective tissue were dissected away, and the skins were spread and dried over a warm plate. The dry skins were extracted overnight with cold benzene, three

or four times, and the pooled extracts were brought to a known volume for absorption analysis with a Hilger Spekker Spectrophotometer. The extinction coefficients $K_{390} = 13{\cdot}0$ and $K_{370} = 11{\cdot}1$ for 1 mg. benzpyrene in 1 ml. benzene in a layer of 1 mm. thickness were used for the estimation.* The light source was a 12-volt projection tungsten lamp with a colour temperature of 2800° K.

The initial amount of benzpyrene on the skin was 0·27 mg. Table II shows the decrease after various periods of contact before killing the mouse, (*a*) when the painted skin was extracted unaltered, and (*b*) when the hair was carefully shaved before extraction.

TABLE II.—*Benzpyrene (mg.) Extractable with Benzene.*

Time in hours	¼	1.	2.	6.	24.	48.
Not shaved (*a*)	0·26	0·19	0·15	0·12	0·08	—
Shaved (*b*)	0·096	0·084	—	0·051	0·01	*nil*

It is evident that after 1 to 2 days there is practically no benzpyrene which is extractable with cold benzene left in the shaved skin. Nevertheless section C shows that the blue-fluorescent compound persists in the skin for a much longer time; evidently this compound is not extracted with benzene.

C. *Attempts to Determine the Nature of the Blue-fluorescent Compound in the Skin of Mice.*

Skins treated as in the previous experiments, after extraction with cold benzene overnight, were mounted in xylene between glass plates and spectrographed. Skins from ¼ to 48 hours after painting showed molecularly dispersed benzpyrene, but above 6 hours the amount was extremely small. Possibly these small amounts could be removed by prolonged treatment with benzene.

The skins were now air-dried to remove all xylene, and then immersed in 0·01 per cent. HCl overnight; the acid, which did not fluoresce, was discarded, and the skins, after drying, were extracted with a known volume of ether. The ether phase fluoresced weakly violet in specimens up to 6 hours and almost pure blue in the 1- and 2-day specimens. The extracted skins emitted only the natural fluorescence.

The ether solutions were next shaken with 0·1 normal NaOH. In the 1- and 2-day specimens the alkaline solution fluoresced blue and the ether did not fluoresce. In the ¼- to 6-hour specimens a little violet fluorescence was left in the ether, corresponding to 0·0002 to 0·00007 mg. These small amounts of molecular benzpyrene can easily be accounted for as left in the skin after the benzene extraction. To remove them completely the alkaline extract was acidified with 0·1 normal H_2SO_4 and extracted with ether, which then fluoresced pure blue.

It may be concluded that the blue-fluorescent benzpyrene is linked with a compound having acid properties.

In another experiment two white mice were killed two days after painting. The dried skins were extracted with benzene and then left in 0·01 normal NaOH overnight. The alkaline solution showed the typical blue bands, but the extracted skins also fluoresced blue, showing that the extraction was incomplete. The alkaline solution was treated successively with half-saturated and saturated ammonium sulphate and the precipitates collected separately. The first (globulin) fraction fluoresced blue; the second (albumin) showed only the natural fluorescence. The "blue" compound was therefore either combined with or adsorbed on the first precipitate.

* Jones (1942) gives for the absorption maxima of benzpyrene in ethanol at 3850 and 3655 A the extinction coefficients 11·9 and 9·5 (reduced to our units) respectively.

H. *The Blue Fluorescence Appears Locally in the Painted Skin.*

In order to test whether the blue-fluorescent compound was produced directly from the benzpyrene painted upon the skin, or indirectly from the hydrocarbon which, according to section F, is quickly absorbed into the blood-stream, the following experiments were made:

(*a*) Mice were painted on both flanks but at different times, so that when killed, comparable skins of different periods of contact were available. These periods were 26 and 2 hours. Hence, after painting the first flank the benzpyrene was circulating for 24 hours in the blood-stream before the second flank was painted.

(*b*) In another set of experiments the second flank was not painted at all.

It was found that in both sets the skin of the flank which was in contact with benzpyrene for 26 hours fluoresced pure blue after extraction with benzene. In (*a*) the other flank (only 2 hours' contact) fluoresced weakly violet, and in (*b*) no benzpyrene fluorescence at all was found in the skin of the second (unpainted) flank.

The experiments have established that the production of the blue-fluorescent compound occurs locally at the painted area.

DISCUSSION.

It is evident that violet-fluorescent benzpyrene is transformed slowly into a blue-fluorescing compound in the living skin of mice. The blue reaches a maximum after a single painting in 4–6 days, then gradually diminishes, but is still detectable after 3 weeks; it could not be found after 5 weeks.

This transformation of violet to blue occurs locally, and is not found in the unpainted parts of the skin of painted animals when precautions against contamination are taken. The chemical nature of the blue-fluorescent compound has not been established owing to the extremely small amounts present in the skin; nevertheless some of its properties can be described.

When the skin is extracted with benzene and afterwards treated with dilute NaOH the blue-fluorescent compound passes into the alkaline solution, and from this it is not removed by shaking with ether. However, some blue fluorescence is left in the skin even after extended contact with NaOH. On precipitating the alkaline solution with half-saturated and saturated ammonium sulphate, the globulin fraction fluoresces blue and not the albumin fraction.

When the skin is treated with dilute HCl after extraction with benzene, and then after drying extracted with ether, the blue-fluorescent compound enters the ether phase. The blue can be transferred to aqueous alkaline solution by shaking with alkali; the ether may be left fluorescing violet if traces of molecular benzpyrene are present in the skin. The blue fluorescence can be repeatedly transferred to water or ether by thus changing the pH.

No decrease in the strength of the blue fluorescence was observed even on prolonged exposure to ultraviolet radiation, so that the compound appears to be light-stable. The experiments with naphthacene do not prove beyond doubt that orthorhombic crystals were not present in the skins showing blue fluorescence; there is the possibility that the few naphthacene molecules in the skin might have failed to enter into the benzpyrene crystals had the latter combined with protein or other substances in the skin.

The blue-fluorescent compound of the skin appears not to be identical with the "BPX" of Peacock, which Chalmers and Crowfoot (1941) claimed to have isolated in the free state and identified as a monohydroxy-benzpyrene. In its fluorescence spectrum they observed two maxima, at 465 and 440 mμ., which do not correspond

to the maxima in the spectrum of our blue-fluorescent compound at 450 and 425 mμ. Furthermore, monohydroxy-benzpyrene fluoresces green in alkaline solution, while our compound fluoresces blue. If the blue-fluorescing compound in the skin was derived not locally from the applied benzpyrene but from benzpyrene changed in the liver to " BPX " and absorbed via the intestine into the blood-stream, as suggested by Peacock (1940), then the whole skin of the animal should show the blue fluorescence, which our experiments entirely negative ; it is only the painted region which fluoresces blue.

The behaviour of the blue-fluorescing substance towards acids and alkalis is in favour of its being a phenolic compound, but it is neither 6-hydroxy-benzpyrene nor 4-hydroxy-benzpyrene, both of which fluoresce red in alkaline solution, according to the tests of Chalmers and Crowfoot (1941).

There is further evidence to show that the blue-fluorescing compound in the skin is not related to " BPX," namely, the quite different lengths of the latent periods before each appears. For the occurrence of " BPX " in the bile after inoculation of benzpyrene into the blood, the latent period is no more than a few minutes, whereas for the production of the blue fluorescence in the skin it is about 6 hours (4–6 days to reach the maximum). The latent period before the appearance of blue fluorescence in the lung and liver after inoculation of benzpyrene into the blood is 2–4 hours, in the kidney cortex 2 hours, in the milk 4–6 hours.

A conclusion to be drawn from these experiments is that the occurrence of a blue-fluorescent compound in the skin represents a *slow* transformation of molecular benzpyrene. We showed in section D that this also occurred in the kidney, lung, liver and mammary glands, where the blue fluorescence was found persisting after molecular benzpyrene had disappeared. (In the bile the violet-fluorescent benzpyrene does not appear as a precursor of the blue-fluorescent " BPX.") The appearance of our compound with its typical banded fluorescence spectrum seems to be associated with living cells, and it may well be that it is an oxidized product. Its precipitation with the globulin fraction and its appearance in the proteins of milk suggest a synthesis of proteins with a benzpyrene-prosthetic group.

It is to be noted that the blue fluorescence occurs, apart from the kidney, in just those tissues where tumours are most likely to develop in animals exposed to benzpyrene. The long persistence of the blue fluorescence in the skin suggests that the derivative of benzpyrene to which it is due may be of much greater importance than the parent hydrocarbon, which quickly disappears after painting.

The localization of the blue fluorescence in the skin in the rows of the hair-bulbs indicates that it is confined to the epithelial cells of the skin. It is seen most strongly in the hair-bulbs because of the large number of epithelial cells present there, especially in cases where there is active regeneration of hair ; no doubt it also occurs in the epidermis, or generally in the Malpighian cells, but cannot be seen because the cells are too few to produce a visible fluorescence under the microscope.

SUMMARY.

(1) Fluorescence spectroscopy and microscopical fluorescence observations were used to study the fate of benzpyrene in living animals.

(2) After inoculation of benzpyrene into mice and rabbits the fluorescence of the kidney, lung and liver changes slowly within 2–4 hours from the violet colour characteristic of dissolved benzpyrene to a new blue fluorescence. The milk of lactating mice shows the first signs of blue fluorescence about 4 hours after inoculation. The clearest effects were seen after painting the skin of white mice with benzpyrene solution ; the fluorescence, at first violet, changes to blue about 6 hours after painting, increasing

in strength until the 4th to 6th day, and fading slowly into the natural fluorescence of the skin within 3 weeks.

(3) In the kidney the blue fluorescence is confined to the cortical zone. In the skin of painted mice under the microscope it is seen most strongly in the rows of the hair-bulbs.

(4) The blue-fluorescent compound is produced locally in the painted area of the skin, and is not deposited there from the blood-stream carrying benzpyrene or any derivative of benzpyrene. It has acid properties, and is soluble in ether and in dilute alkali.

(5) Its persistence in the skin over a period of several weeks after one single painting suggests that the blue-fluorescent derivative of benzpyrene is more closely connected with carcinogenesis than the parent hydrocarbon itself, which disappears from the skin within two days. As further evidence, tumours can be produced by benzpyrene in the lung, liver and mammae (though not in the kidney cortex), which are just those sites where the blue-fluorescent compound appears.

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THE FLUORESCENCE OF 3 : 4-BENZPYRENE *IN VIVO*. PART II: THE INTER-RELATIONSHIP OF THE DERIVATIVES FORMED IN VARIOUS SITES.

I. DONIACH, J. C. MOTTRAM AND F. WEIGERT.

From the Physico-Chemical Department of the Mount Vernon Hospital, Northwood, Middlesex.

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IN our previous communication (Doniach, Mottram and Weigert, 1943), we gave evidence that a blue fluorescence spectrum with maxima near 450 and 425 mμ. similar to that of " BPX " appears in the kidney cortex, lung and liver of mice and rabbits, in the milk of lactating mice after intravenous injection of the hydrocarbon, and in the skin of white mice after painting. These sites are important with respect to tumour formation by carcinogenic hydrocarbons. We showed that in the skin the blue fluorescence is due to a derivative of benzpyrene produced slowly from the violet-fluorescent hydrocarbon locally in the painted area itself. In the following experiments we have tried to establish whether the blue fluorescence in the kidney cortex,

liver and lung is also due to a local transformation of benzpyrene in each organ, or whether it is due to re-absorbed " BPX " from the bile. This possibility could not be excluded *a priori* since Peacock (1940) described a blue-fluorescent derivative in the urine, which he assumed to be " BPX" re-absorbed from the intestine.

EXPERIMENTAL.

The problem could be tackled only indirectly, by attempting through fluorescence spectroscopy to trace the pre-history of the appearance of the blue fluorescence in the organs mentioned, and by trying to determine whether or not " bile-BPX " is re-absorbed unchanged from the intestine. The benzpyrene was given the chance to run through various channels in the mice :

(A) Directly through the blood stream by intravenous inoculation.

(B) Indirectly, through absorption from the intestine by feeding.

(C) By intravenous inoculation into mice after having ligated and cut the common bile duct, thus preventing the entrance of molecular benzpyrene and of " BPX " into the lumen of the digestive tract.

(D) By feeding a mouse with benzpyrene after this operation, thus introducing the hydrocarbon into the jaundiced mouse by absorption from the intestine.

(E) By feeding mice with preparations of " BPX."

Other modes of application of benzpyrene, e.g. subcutaneous injection of solutions in fatty solvents, implantation of crystals or pastilles, etc., have not yet been studied, because the amount of hydrocarbon accumulated at the various remote sites would be too small to guarantee an unmistakable identification by fluorescence spectroscopy.

Material and Methods.

Spectrography.—The spectroscopical technique for identifying the violet and blue fluorescences of benzpyrene and its derivatives in biological specimens was described in detail in Section A of our previous paper (1943), in which also photographs of representative spectra were reproduced.

Mice.—Pen-inbred Bagg albinos were used.

Injections.—Fifty-nine mice were inoculated intravenously with 1 c.c. of 0·05 per cent. coarse suspension of benzpyrene in saline.

Feeding.—Sixty-two mice were fed by introducing with a syringe and a smoothed-off serum needle direct into the stomach 0·5–1·0 c.c. of benzpyrene suspension thickened with a little flour and in some cases additional bile salts. Five mice were fed with samples of " BPX " from other animals.

Operation.—In 23 mice the common bile duct was divided between two ligatures under ether anaesthesia. The mice stood the operation well, showing clinical jaundice in 24 hours. The gall bladders showed enormous distension after a few days.

Collection and treatment of material.

Blood.—The mice were decapitated under deep ether anaesthesia and either whole or citrated blood collected from the neck vessels. The serum or plasma was separated from the red cells and spectrographed. Samples of blood were also collected from the tails of living mice.

Intestines.—The alimentary tract was removed as one tube from the stomach to the rectum and fixed in formol-saline. After being cleaned as far as possible from adherent fat, its wall and contents were spectrographed at various points along its length.

Other organs.—The gall bladder, kidneys, lungs, liver, brain, fat, and in a few cases skin and spleen were removed separately and their fluorescences spectrographed.

TABLE I.—*Fluorescence Phenomena Recorded by Spectroscopy in Mice Treated with Benzpyrene (A–D) and "BPX" (E).*

	Normal mice.	Mice whose common bile duct was ligated and cut.	Normal mice.

TABLE I.—*Fluorescence Phenomena Recorded by Spectroscopy in Mice Treated with Benzpyrene (A–D) and "BPX" (E).*

Site.	Normal mice. A. Intravenous inoculation (53 mice).	Normal mice. B. Feeding (45 mice).	Mice whose common bile duct was ligated and cut. C. Intravenous inoculation (6 mice).	Mice whose common bile duct was ligated and cut. D. Feeding (17 mice).	Normal mice. E. Feeding with "BPX" (5 mice).
Plasma	*Violet* for about 1 hour, followed by *whitish-blue* reaching a maximum at about 6 hours and disappearing after about 24 hours. Whitish-blue persists after extraction with ether. (No blue.)	As column A.	*Violet* at first. Then *blue* persisting for 5 days, diminished after extraction with ether (ether blue).	As column C, but less intense.	*Nil.*
Bile	*Blue* "BPX" (no violet precursor) disappearing after 24 hours.	As column A.	Traces of *blue* "BPX" in greatly distended gall bladder.	As column C.	*Nil.*
Stomach	*Contents* / *Wall* } *Nil.*	*Contents : Violet*, disappearing after 1 day. *Wall :* After ether extraction, *nil.*	*Contents : Nil.* *Wall :* After ether extraction, *blue.*	*Contents : Violet.* *Wall :* After ether extraction, *blue.*	*Contents* / *Wall* } Traces of *blue.*
Small intestine	*Contents : Blue*, reaching maximum after about 6 hours, then disappearing. *Wall:* After ether extraction, *blue* where "BPX" adherent to lining.	*Contents : Violet*, advancing at about 20 cm. per hour, followed by *blue* with maximum at about 6 hours. Advancing violet slowly disappears. *Wall :* As column A.	*Contents : Nil.* *Wall :* After ether extraction, *blue.*	*Contents : Violet*, advancing more slowly than in B. (No blue.) *Wall :* After ether extraction, *blue.*	*Contents* / *Wall* } Traces of *blue.*
Large intestine	From about 2 hours : *Contents : Blue-green* disappearing after 24 hours. (No blue.) *Wall :* After ether extraction, *blue-green* adherent to lining.	From about 3 hours : *Contents : Blue-green* + *violet*, slowly disappearing. *Wall :* As column A.	*Contents : Nil.* *Wall :* After ether extraction, *blue.*	*Contents :* Weak *violet.* *Wall :* After ether extraction, *blue.*	*Contents* / *Wall* } Traces of *blue-green.*
Fat	*Violet.*	*Violet.*	*Violet.*	*Violet.*	*Nil.*
Kidney cortex	After 1 hour increasingly *blue*, disappearing after 1–2 days.	As column A.	*Violet*, followed by stronger and slightly different *blue* from that of A and B.	As column C.	,,
Liver	*Violet*, after 2 hours increasingly *blue*, disappearing after 1 day.	As column A.	*Violet*, then stronger *blue* than in A and B.	As column C.	,,
Lung	*Violet*, + yellow due to trapped particles of benzpyrene ; some *blue* after 2 hours.	*Violet ;* some *blue* after 2 hours.	*Violet*, then stronger *blue* than in A and B.	As column C.	,,
Brain	*Violet.*	*Violet.*	*Violet.*	*Violet.*	,,
Spleen	*Nil.*	*Nil.*	*Blue.*	*Blue.*	,,

Additional treatment.—The fluorescences of ethereal and watery extracts of the various organs and fluids were also spectrographed.

Not every mouse was subjected to this thorough examination; only the fluorescences of the tissues relevant to the experiment in hand were spectrographed, but all the opened mice were examined superficially under the ultraviolet lamp.

RESULTS.

The fluorescences characterizing the various organs and body fluids of the various groups of animals are brought together in Table I, in which "violet" stands for the fluorescence spectrum of molecular benzpyrene with its typical sharp maximum at 427 mμ. and the diffuse maximum at about 455 mμ.; "blue" stands for the fluorescence spectrum with diffuse maxima at about 450 and 425 mμ., resembling "BPX"; "blue-green" stands for the spectrum with diffuse maxima near 440 and 465 mμ. resembling "BPF" (Chalmers, 1938); and "whitish-blue" stands for a fluorescence spectrum with one broad maximum near 460 mμ., simulating a greatly enhanced natural fluorescence of the skin.* These spectra are illustrated in Fig. 1 of our previous communication.

DISCUSSION.

On comparing column A with column B and column C with column D in the table it is evident that feeding is equivalent to an intravenous inoculation in normal mice as well as in mice whose common bile duct was ligated and cut. Indeed, feeding seems to be the mildest method for repeatedly introducing large doses of benzpyrene into a mouse, the hydrocarbon in the small intestine acting as a store from which it is quickly† absorbed into the blood stream. This store is equivalent to the benzpyrene particles which are trapped in the lung capillaries after intravenous inoculation. Blue-fluorescing "BPX"—whether produced by the mouse (columns A and B of the table) or fed to it (column E)—present in the lumen of the intestine is changed abruptly to blue-green-fluorescent "BPF" on entering the caecum.

The most striking difference between the fluorescence phenomena in normal mice and in those whose common bile duct has been ligated and cut is seen in the ether-extracted wall of the digestive tract. In normal mice only that part fluoresces blue which has been in contact with "bile-BPX" inside the lumen. In the other mice the entire length of wall from stomach to rectum fluoresces blue, although its lumen has been absolutely free from "bile-BPX." Hence this blue fluorescence cannot have any other origin than a "vital fluorescence-staining" by the blue-fluorescing compound accumulated in the plasma under the conditions following obstruction of the common bile duct. This staining of the walls of the stomach and colon can be taken, therefore, as an indicator for the presence in the blood stream of a blue-fluorescent benzpyrene derivative which may be called "plasma-BPX." Such a staining occurs also in the spleen (possibly owing to its large content of free blood), but not in the brain, skin or adipose tissue. This staining is therefore selective. The increased blue fluorescence of the kidney cortex, liver and lung indicates that these tissues are also stainable. In normal mice the *unstained* wall of the stomach and colon shows that the blue fluorescence of the kidney cortex, liver and lung after inoculation or feeding of benzpyrene

* This bright whitish-blue fluorescence, which has not yet been thoroughly investigated, appears only in the plasma after treatment with benzpyrene. It is apparently not due to an impurity (having a similar fluorescence), which according to Berenblum and Schoental (1942) is usually present in benzpyrene.

† It could be proved by photometric methods similar to those described in Section F of our previous communication that out of 0·028 mg. of benzpyrene introduced by feeding, 0·02 mg., i.e. about 70 per cent., had been absorbed from the small intestine within 4 hours.

can *not* be due to a staining effect. The only alternative is that in these organs the blue fluorescence must be ascribed to a local transformation from violet-fluorescent benzpyrene. Further evidence that " BPX " is not absorbed into the blood stream from the intestine is provided by experiment E, in which, although " BPX " was directly fed to normal mice, it could not be detected in the blood or any parenteral organ.

Three different origins of blue fluorescence with bands near 450 and 425 mμ. can therefore be distinguished in mice inoculated or fed with benzpyrene :

(1) "*Tissue-BP-blue*," benzpyrene derivatives produced locally and relatively slowly from molecular benzpyrene in the kidney cortex, lung, liver, Malpighian layer of the skin and probably in the mammary glands. Since these (except the kidney) are the sites of tumour production by carcinogenic hydrocarbons, we are especially interested in the chemical nature of the blue-fluorescing derivatives in these organs.

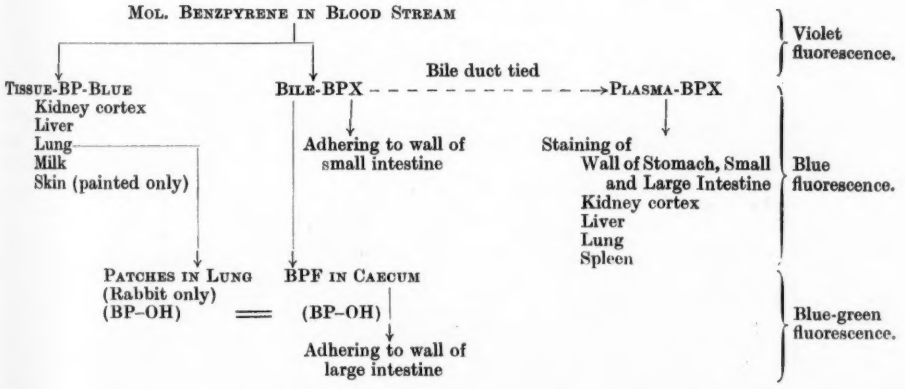
(2) "*Bile-BPX*," which is produced quickly, flows into the duodenum under normal conditions, is fixed by the lining of the small intestine, and is not re-absorbed from the gut into the blood stream.

(3) "*Plasma-BPX*," which appears in obstructive jaundice and selectively stains certain organs so that they show a blue-banded fluorescence.

CONCLUSIONS.

Our main findings on the fluorescence of benzpyrene *in vivo* from this and the preceding paper are summarized in the accompanying chart.

Chart showing the Inter-relationship of the Various Fluorescent Derivatives of Benzpyrene Produced in vivo. *Data taken from Parts I and II.*



The chart shows that a relation exists between " tissue-BP-blue," " bile-BPX " and " plasma-BPX," which is possibly due to the linking of the same blue-fluorescent benzpyrene derivative as a prosthetic group to various cell constituents. The relation between " bile-BPX " and " plasma-BPX " is clear, since the latter appears in mice only when the normal excretion of " bile-BPX " is prevented by tying the common bile duct. On the other hand, the relation between " bile-BPX " and " tissue-BP-blue " needs further comment.

We have recorded the striking and abrupt change of the blue-fluorescing small

intestinal contents to blue-green-fluorescing faeces in the caecum and onwards. From the faeces of rats Chalmers and Crowfoot (1941) isolated a monohydroxy-benzpyrene with the same blue-green fluorescence. Since Chalmers and Peacock (1941) were able to prepare the same substance from blue-fluorescent " bile-BPX " by treatment with alkali and adsorption on alumina, they claimed to have established that the two derivatives, which had been earlier described by Chalmers (1938) as " BPF " (faeces) and " BPX " (bile), were chemically identical. We submit two possible explanations for the abrupt change in fluorescence at the caecum :

(1) That the different fluorescence spectra indicate two chemically different derivatives of benzpyrene, the blue-green-fluorescent derivative being present in only small amount proximal to the ileo-caecal valve and in larger amount in the caecum and onwards.

(2) That the abrupt transition in fluorescence indicates a purely physical change, which has yet to be studied (Weigert, 1941).

We mentioned in the previous paper (section D) that in rabbits inoculated intravenously with benzpyrene the lungs sometimes showed blue-green fluorescent patches. These patches showed the same chemical and fluorescent properties as " BPF " in mouse faeces. The rabbit lung patches could not have had any other origin than " tissue-BP-blue." Thus, " bile-BPX " and " tissue-BP-blue " are both liable to a transformation into the same blue-green-fluorescent benzpyrene derivative.

SUMMARY.

Mice were fed and inoculated intravenously with benzpyrene, and an attempt was made to trace the origin of the blue fluorescence which appeared in the lungs, liver and kidneys. It was found to be due to locally changed benzpyrene, not to " bile-BPX " re-absorbed from the intestines.

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THE PATH OF DISSEMINATION OF RABIES VIRUS IN THE BODY OF NORMAL AND IMMUNIZED MICE.

I. J. KLIGLER AND H. BERNKOPF.

Department of Hygiene, The Hebrew University, Jerusalem.

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IT has been generally assumed that rabies virus extends along the nerve paths to the central nervous system. This assumption is based on post-mortem studies showing the localization of the virus at the time of death (Sabin and Ruchman, 1940; Leach and Johnson, 1940). To our knowledge there is one experimental study (Goodpasture, 1925) in which the author attempted to follow the path of distribution of the virus from the time of infection to death. Goodpasture employed the histological technique, which gave rather inconclusive results. The author summarizes his findings as follows: "It is believed that the virus enters the central nervous system through nerves supplying the inoculated muscle." The purpose of our present study was to ascertain the localization and dissemination of virus following various routes of infection in normal and immunized mice.

EXPERIMENTAL.

In the first series of experiments groups of mice were infected intraperitoneally or subcutaneously, and at stated intervals two of the infected mice were killed and various tissues examined for the presence of virus. The tissues were removed aseptically, weighed, ground with sand in a mortar and suspended in saline. As a rule 0·03 c.c. of a 1 : 10 suspension of tissue was inoculated intra-cerebrally into each of three

TABLE I.—*Dissemination of Virus in Various Tissues of Mice Infected by the i.p. and s.c. Routes, respectively.* (*3-week-old mice were used.*)

Mode of infection.	Surviving mice.	Tissue examined.	Hours after infection.					
			3.	6.	24.	72.	96.	120.
0·5 c.c. of a 1 : 50 brain emulsion i.p.	6/6	Peritoneal washings	3/3	3/2	3/0	3/0	3/0	3/0
		Inguinal and axillary glands	3/3	3/3	3/0	3/0	3/0	..
		Liver and spleen	—	3/3	3/0	2/0	3/0	3/0
		Lower cord	—	3/0	3/0	3/0	3/3	3/3
		Upper cord	—	3/0	2/0	3/1	1/1	3/3
		Brain	—	3/0	3/0	3/0	3/3	3/3
0·5 c.c. of a 1 : 50 brain emulsion s.c. on the back	6/6	Peritoneal washings	—	—	—	—	—	—
		Inguinal and axillary glands	3/3	3/1	3/0	3/0	3/0	3/0
		Liver and spleen	3/0	3/0	3/0	3/0	3/0	3/0
		Lower cord	—	3/0	3/0	3/0	3/3	3/3
		Upper cord	—	3/0	3/0	3/1	3/3	3/3
		Brain	—	2/0	3/0	3/0	3/3	3/3

— = Not examined.

Numerator = Number inoculated. Denominator = Number died of typical infection.

mice, and the presence and concentration of virus determined by the number of mice developing the infection.

Experiment 1: In this experiment 3 weeks old mice were used. One set received 0·5 c.c. of 1 : 50 brain emulsion intraperitoneally and another set 0·5 c.c. of the same material subcutaneously. Groups of mice were killed at the end of 3 hours, 6 hours, etc., and the tissues examined. The mice remaining after 120 hours served as controls. In each group six mice remained and all of them died with typical paralysis. The results are summarized in Table I.

In a second experiment the mice were infected subcutaneously, one set in the hind and the other in the fore limb. In this experiment 0·2 c.c. of a 1 : 20 brain emulsion was injected. The rest of the procedure was the same as in experiment 1. The results obtained in this experiment are shown in Table II.

TABLE II.—*Dissemination of Rabies Virus in Mice Infected in the Fore and Hind Leg respectively.*

Mode of infection.	Surviving mice.	Tissue examined.	Hours after infection.					
			4.	24.	72.	96.	120.	168.
0·2 c.c. of a 1 : 20 brain emulsion s.c. in fore leg	5/5	Muscles (1)	3/3	3/0	—	—	—	—
		Inguinal and axillary glands	3/3	3/0	—	—	3/2	3/1
		Mesenterial gland	3/0	3/0	—	—	3/0	3/1
		Liver and spleen	3/0	2/0	—	—	—	—
		Lumbar cord	—	—	3/0	3/1	3/3	—
		Thoracic cord	—	—	3/1 ?	3/3	3/3	—
		Cervical cord	—	—	3/3	3/3	3/3	—
		Brain	—	—	—	3/3	3/3	—
0·2 c.c. of a 1 : 20 brain emulsion s.c in hind leg	5/5	Muscles	3/3	3/0	—	—	—	—
		Inguinal and axillary glands	3/0	3/0	—	—	3/0	3/2
		Mesenterial gland	3/0	3/0	—	—	3/0	3/1 ?
		Liver and spleen	3/0	3/0	—	—	—	—
		Lumbar cord	—	—	3/1	3/3	3/3	—
		Thoracic cord	—	—	3/1 ?	3/3	3/3	—
		Cervical cord	—	—	3/0	3/1	3/3	—
		Brain	—	—	—	3/0	3/3	—

— = Not examined. (1) = Muscle at site of inoculation.
Numerator = Number inoculated. ? = Doubtful symptoms not typical.
Denominator = Number died of typical infection.

These experiments were repeated with minor variations with fairly uniform results. Eight experiments were made with the peritoneal and subcutaneous method of infection. The results showed that after intraperitoneal infection virus could always be recovered from the peritoneum during the first 6 hours after the inoculation, but was no longer demonstrable after 24 hours. The washing was made by making an opening in the abdomen, admitting two doses of saline, each time 0·5 c.c., stirring about with the pipette and then withdrawing the fluid. The test mice were given 0·03 c.c. of these washings i.c. As a rule virus could also be recovered from the spleen and liver during the first 6 hours but not after 24 hours. The mesentery and peripheral glands showed the presence of virus quite regularly after 6 hours and on no occasion after 24 hours. Beginning with 24 hours no virus could be detected in any organ or tissue tested until 72 to 96 hours after the infection, when virus was found only in the nerve tissues. It is of interest to note that after intraperitoneal infection the earliest appearance of virus was always in the cervical cord, and only subsequently in the brain and lastly in the lumbar cord.

A summary of these experiments is given in Table III.

TABLE III.—*The Distribution of Rabies Virus in Various Tissues of Mice Infected Intraperitoneally at Various Intervals.*

Material tested.	Hours after infection.							
	3.	6.	24.	48.	72.	96.	120.	172.
Peritoneal washings	+++	+++	−−	−	−−	−	−	−
	++++	+++	..	..	..	..	..	..
Liver and spleen	+++	+++	−−−	−+	−−−	−−−	−−	−
	++	++−	−−	..	−	..	..	..
Mesenterial glands	++	+−−	−−−	−	−−	−	−	+
Inguinal and axillary glands	++−	+−+	−−	−	−	−	..	..
Lumbar cord	−−	−	−−	−−	−−	−−	++	+
Thoracic cord	−−	−−	−−	−−	−−	+−	..	..
Cervical cord	−−	−−−	−−−	−−	−−−+	+++	+++	+
"	..	..	−−	..	−−−−	−+−	..	..
Brain	−	−	−−	..	−−	+−	++	+

\+ = positive finding
− = negative „ } Each sign is the result in one experiment.

Infection in the leg muscles gave somewhat different results. In this procedure, too, the virus was recoverable at the site of inoculation within 4–6 hours, but only once 24 hours after the infection. By this procedure however the virus was recovered from lymphatic glands only on two occasions and then only in those proximal to the point of inoculation; immediately after the inoculation there was no general lymphatic distribution as was the case with peritoneal inoculation. Nor was virus recovered from the liver and spleen. Only after the infection had progressed to paralysis (7 days) was virus recovered again at the site of inoculation as well as in the lymphatic glands; apparently after the virus has multiplied sufficiently it spreads centrifugally. Of further interest is the first appearance of virus in the cord; in animals infected in the fore-leg the virus was first recovered from the cervical cord, 3 days after infection; in those infected in the hind leg the lumbar and thoracic segments of the cord were the first to be involved, also 3 days after the infection. In the former case the spread was to the brain and thoracic cord, while in the latter the direction of spread was naturally to the cervical cord and brain. These data are summarized in Table IV.

Effect of Age on Path of Distribution of Virus.

Previous observations have shown that young mice were more susceptible to intraperitoneal infection with rabies virus than older ones. Consequently we tried to ascertain whether there was any noticeable difference in the distribution of the virus in 3 weeks and 3 months old mice respectively.

Two sets of mice were given an 0·5 c.c. of 1 : 50 suspension of infected mouse brain and the organs and tissues tested at intervals as in the preceding experiments. These tests were made daily up to 4 days; after that time the surviving mice were observed for symptoms and fatalities. As previously noted at least 90 per cent. of the 3 weeks old mice died of a typical infection, whereas among the older mice the fatality was 60 per cent. or less.

The experiment shown in Table V indicates the course of infection in the two sets of mice. It will be noted that during the first 6 hours the virus is more or less equally disseminated in the two groups of mice. In both groups the virus disappears from the visceral organs and lymphatics, to reappear on the 4th day in the cord in the young mice, but not in the older ones. If older mice succumb to the infection then the virus is found in the cord as in the young mice. Failure to appear in the cord suggests that the virus was destroyed before it established itself. It may be noted that in this

as in other experimental intraperitoneal infections the upper part of the cord is the first invaded, indicating that the nerve invasion takes place in the peritoneal cavity and not in the glands, since virus is found both in the inguinal and axillary glands.

TABLE IV.—*Dissemination of Rabies Virus in the Body of Mice Infected in the Fore and Hind Leg Muscle respectively.*

Material tested.	Hours after inoculation. 4.	24.	72.	96.	120.	168.
Hind leg :						
Muscles	++	−−	+	..	..	−
Inguinal and axillary glands	−	−	..	..	−	+
Inguinal glands	−	−	−	−	−	−
Axillary glands	−−	−	..	−	−	+
Mesenterial glands	−−	−−	..	−	−	++
Liver and spleen	−	−	..	..	..	..
Lumbar cord	..	..	+−+−−	+−+++	+++++	..
Thoracic cord	..	..	+?+−−+	+++++	+++++	..
Cervical cord	..	..	−−−−−	+−+++	++++	..
Brain	..	..	−−−	−++	+++	..
Fore leg :						
Muscles	++	−−	−	..	..	+
Inguinal and axillary glands	+	−−	−	..	+	+
Inguinal glands	−	−	..	−	−	−
Axillary glands	++	−	..	−	−	−
Mesenterial glands	..	..	..	..	..	..
Liver and spleen	−−	−	..	..	..	..
Lumbar cord	..	..	−−−−−	+−++?−	++++	..
Thoracic cord	..	..	+?−−−−	+−+++	+++++	..
Cervical cord	..	..	+−+?−+	+++++	+++++	..
Brain	..	..	−−	+++	+++	..

Signs see Table III.

TABLE V.—*The Dissemination of Rabies Virus in 3-week and 3½-months Old Mice, after Intraperitoneal Infection.*

Age of mice.	Infecting dose.	Material examined.	Remaining mice.	Hours after infection. 3.	6.	24.	72.	96.
3 weeks	0·5 c.c. of 1 : 50 brain emulsion i.p.	Washings	9/8	3/3	3/2	−	−	−
		Inguinal and axillary glands		3/0	3/2	3/0	−	−
		Mesenterial glands		−	3/0	3/0	−	−
		Liver and spleen		3/3	3/0	3/0	−	−
		Lower cord		−	−	−	3/0	1/0
		Upper cord		−	−	−	3/0	3/3
		Brain		−	−	−	3/0	3/0
3½ months	0·5 c.c. of 1 : 50 brain emulsion i.p.	Washings	8/5	3/3	3/3	−	−	−
		Inguinal and axillary glands		3/3	3/1	3/0	−	−
		Mesenterial glands		−	3/1	3/0	−	−
		Liver and spleen		3/1	3/3	3/0	−	−
		Lower cord		−	−	−	3/0	3/0
		Upper cord		−	−	−	3/0	2/0
		Brain		−	−	−	3/0	2/0

Signs see Table II.

Distribution of Virus in Immunized Animals.

We next directed our attention to the dissemination of virus in animals previously immunized in comparison with controls infected at the same time and in the same manner.

In the first series of experiments groups of mice were given one, two or five injections of 0·25 c.c. of a formalized mouse brain or culture vaccine (Kligler and Bernkopf, 1938), respectively. The immunizing injections were given at intervals of 3 days, and were so arranged that the groups receiving only one dose of vaccine received the injection at the same time as the others received their final dose. The intraperitoneal test infection was given two weeks after the final immunizing dose.

In this series we examined only the peritoneal washings and liver and spleen, the object being to ascertain the immediate fate of the virus injected into immunized animals.

The results of a typical experiment are shown in Table VI.

TABLE VI.—*Recovery of Virus in Immunized and Normal Mice.*

Method of immunization.	Immune test. i.p.	Three hours after infection.				Six hours after infection.			
		Washings. 1 : 10.	Washings. 1 : 100.	Liver, spleen. 1 : 10.	Liver, spleen. 1 : 100.	Washings. 1 : 10.	Washings. 1 : 100.	Liver, spleen. 1 : 10.	Liver, spleen. 1 : 100.
Controls (3 weeks old)	4/4	3/3	3/3	3/2	3/3	3/3	3/3	3/3	3/1
1 × 0·25 c.c. culture vaccine i.p.	5/4	3/3	3/2	3/2	3/2	3/3	3/1	2/0	3/0
2 × 0·25 „ ditto	4/0	3/3	3/3	3/3	3/2	3/3	3/1	2/0	3/0
5 × 0·25 „ „	5/0	3/1 ?	3/1 ?	3/0	3/0	3/1	2/0	3/0	3/0
1 × 0·25 „ brain vaccine i.p.	5/1	3/3	3/3	3/3	3/0	3/1 ?	3/1	3/3	3/0
2 × 0·25 „ ditto	5/0	3/3	3/3	3/1	3/1 ?	3/0	3/0	3/0	3/0
5 × 0·25 „ „	5/1 ?	3/0	3/0	3/1	2/1	3/0	3/0	3/0	3/0

Signs see Table II.

Other experiments gave practically the same results. In one experiment the glands were also examined, and a small amount of virus recovered 3 but not 6 hours after the infection.

It will be noted that virus was recovered from the control animals, in a fairly high concentration, 3 and 6 hours after the infection. In the immunized animals the virus disappeared either in 3 or 6 hours according to the number of immunizing doses, or, in other words, the degree of immunity.

In a second series of experiments groups of mice were given one, two and five doses of 0·25 c.c. of a formalized mouse brain vaccine and then infected in the hind or fore leg and the fate of virus followed. The mice receiving only one immunizing dose reacted like normal mice ; the mild degree of immunity had no effect on the dissemination of virus, and all remaining animals succumbed to the infection.

Those receiving two immunizing doses gave variable results, some developing the infection, others not. Of the remaining mice (not killed for determining localization of virus) all of the controls died, while of those immunized, 2 out of 4 receiving the infection in the fore leg and 2 out of 7 receiving the infection in the hind leg succumbed to the disease.

More consistent results were obtained in the mice receiving five immunizing doses. Not all the mice proved immune to the subcutaneous infecting dose ; 6 of 11 receiving the infection in the hind leg and 1 of 11 receiving the infection in the fore leg died, as against all of the controls. Details of one experiment are given in Table VII.

DISCUSSION.

Relatively little is known of the fate of rabies virus injected into an animal by diverse routes. The studies reported above bring to light a number of interesting points. After intraperitoneal injection into mice the virus is recovered during the

TABLE VII.—*Fate of Rabies Virus Injected in Legs of Normal and Immunized Mice.*

Mode of immunization and subsequent infection.	Tissue examined.	Hours after infection. 6.	24.	72.	96.	120.	168.
5 × 0·25 c.c. brain vaccine + 0·1 c.c. 1 : 10 brain emulsion in hind leg	Muscles	3/3	3/0	3/0	—	—	3/0
	Inguinal glands	3/0	3/0	—	3/0	3/0	3/0
	Mesenterial glands	3/0	3/0	—	3/0	3/0	—
	Axillary glands	3/0	—	—	—	—	3/0
	Lumbar cord	—	—	3/0	3/0	3/0	—
	Thoracic cord	—	—	3/0	3/0	3/0	—
	Cervical cord	—	—	3/0	3/0	—	—
	Whole cord	—	3/0	—	—	—	—
5 × 0·25 c.c. brain vaccine + 0·1 c.c. 1 : 10 brain emulsion in fore leg	Muscles	3/3	3/0	3/0	—	—	3/0
	Inguinal glands	3/0	3/0	—	3/0	3/0	3/0
	Mesenterial glands	3/0	..	—	—	—	—
	Axillary glands	3/0	3/0	—	3/0	3/0	3/0
	Lumbar cord	—	—	3/0	3/0	3/3	—
	Thoracic cord	—	—	3/0	3/0	3/3	—
	Cervical cord	—	—	3/0	3/0	..	—
	Whole cord	—	3/0	—	—	—	—
Normal, 3-weeks-old mice, 0·1 c.c. 1 : 10 brain emulsion in hind leg	Muscles	3/3	3/0	3/1	—	—	3/0
	Inguinal glands	3/0	3/0	—	3/0	3/0	3/0
	Mesenterial glands	3/0	—	—	3/0	3/0	—
	Axillary glands	3/0	3/0	—	—	—	3/3
	Lumbar cord	—	—	3/0	3/1	3/2	—
	Thoracic cord	—	—	3/2	3/2	3/2	—
	Cervical cord	—	—	3/0	3/1	3/2	—
	Whole cord	—	3/0	—	—	—	—
Normal, 3-weeks-old mice, 0·1 c.c. 1 : 10 brain emulsion in fore leg	Muscles	3/3	3/0	3/0	—	—	3/2
	Inguinal glands	3/0	3/0	—	3/0	3/0	3/0
	Mesenterial glands	3/0	..	—	—	—	—
	Axillary glands	3/2	3/0	—	3/0	3/0	3/0
	Lumbar cord	—	—	3/0	3/0	3/3	—
	Thoracic cord	—	—	3/0	3/3	3/3	—
	Cervical cord	—	—	3/3	3/3	—	—
	Whole cord	—	3/0	—	—	—	—

Signs see Table I.

first 6 hours not only from the peritoneal cavity, but also from the liver, spleen, axillary and inguinal glands. Apparently its subsequent invasion of the nervous system is not by way of the nerves supplying these organs. If it were, it should later be found simultaneously in different parts of the cord, in the lower as well as in the upper segments. As a matter of fact after peritoneal infection the cervical cord is always the first segment invaded, thus indicating some pathway of predilection of the virus from the peritoneal cavity to the cord. A great part of virus injected intraperitoneally seems to be destroyed in various glands during the first 24 hours; the widespread lymphatic distribution of the virus and its subsequent destruction offer a possible explanation of the relative insusceptibility of older mice to this route of infection, as well as for the better immunizing effect of intraperitoneal as compared with subcutaneous virus. The spread of subcutaneous virus is limited; it is recovered during the first 6 hours only from the lymph glands near the site of infection.

After 24 hours, whatever the site of infection, no virus is found in any of the organs tested. These findings parallel the observations of Webster and Clow (1936) regarding the fate of intraperitoneally injected encephalitis virus. The small amount of virus actually invading the nerves seems to need up to 72 hours for multiplication and extension along the nerve path before it has reached the cord and is present there in detectable amounts. Once having established itself in one part of the cord, it spreads rapidly from there, and within 24 hours is found in all other parts of the central

nervous system, brain as well as cord. At this time there is as yet in the mice no clinical sign of the infection; the first paralytic symptoms do not appear before 144–168 hours. This considerable time interval between the actual invasion of the central nervous system and the appearance of clinical signs of illness finds again a parallel in the observations of Webster in encephalitis virus infection.

After 144–168 hours the virus spreads centrifugally as shown by its presence in various lymph glands. There appears to be no connection between the portal of entrance of the virus and its reappearance in certain glands. When the animal lives long enough the centrifugal spread of the virus is more general. It seems, therefore, that virus distribution at the time of death depends on chance and may vary from animal to animal. This may explain the different distribution of virus in single human cases, as reported by Sabin and Ruchmann (1940) and by Leach and Johnson (1940).

In immunized animals the virus disappears more rapidly from the site of infection and from the lymphatic glands than in normal ones, a connection existing between the degree of immunity and the rate of disappearance of the virus. If immunity is not adequate to prevent the virus from reaching the cord, no difference is observed in the rate of spread of the virus to the other parts of the central nervous system in immunized as compared with normal mice; the mechanism of immunity consists apparently in a rapid destruction of the virus during the first hours after infection.

SUMMARY.

1. Rabies virus injected intraperitoneally in mice is demonstrable during the first 6 hours in the peritoneal washings, the mesentery, axillary and inguinal glands. Beginning with 24 hours after the infection no more virus is demonstrable; it reappears after 72 hours in the upper cord, spreading from there upwards and downwards to the other parts of the central nervous system during the following 24 hours. In one instance virus reappeared after 172 hours in the mesentery glands, suggesting centrifugal spread.

2. Rabies virus injected subcutaneously in mice is demonstrable after 4 hours, but not after 24 hours in the muscles at the site of infection, and occasionally in the regional lymph nodes. There is no general lymphatic distribution as after intraperitoneal infection. After 72 hours the virus is present in the cord, the part first invaded depending on the site of infection. After infection in the hind leg virus is first found in the lumbar and thoracic cord; after injection in the fore leg it appears first in the cervical cord. Later on it is found in all parts of the central nervous system. After 168 hours virus was found in various lymphatic glands and, in one case, in the muscles at the site of infection.

3. Age had no apparent effect on the distribution of virus in mice.

4. In immunized mice the virus disappeared more rapidly from the site of infection than in the controls; the rate of disappearance depended on the degree of immunity.

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THE CHLORIDE EXCRETION IN STEATORRHOEA: COMPARISON WITH THE CONDITIONS IN ADDISON'S DISEASE.

F. T. G. PRUNTY AND S. J. R. MACOUN.

From the Department of Pathology, St. Thomas's Hospital Medical School.

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LOEB, Atchley, Benedict and Leland (1933) showed that adrenalectomized dogs excreted excessive amounts of sodium and chloride, and McCance (1936) described experiments, in which sodium and chloride depletion of the body of severe degree were produced, giving rise to symptoms resembling those of Addison's disease. Allott (1936) has also made a comprehensive study of the blood changes in Addison's disease, pointing out the depletion of sodium and chloride that occurs. Anderson and Lyall (1937) carried out estimations of the urinary chloride concentration in specimens taken at the same time as blood for plasma chloride estimation, and found in Addison's disease high urinary chloride concentrations although the plasma chloride level was definitely subnormal. Cutler, Power and Wilder (1938) agreed with this finding, but suggested that more controlled conditions were desirable, and instituted a 52-hour test period during which a diet equivalent to 0·95 g. chloride ion, 0·59 g. sodium ion and 4·1 g. potassium ion was given and supplemented by a dose of potassium citrate equivalent to 33 mg. potassium ion per kilogram body weight on each of the first two days. The present paper demonstrates the need for this greater control, a case of steatorrhoea being described in which the question of the differential diagnosis of Addison's disease was raised.

G. D—, aged 52, a gardener, was admitted to hospital in July, 1942, complaining of attacks of diarrhoea for the last six years. There were remissions of about six months, at the end of which there followed several weeks during which there were attacks of diarrhoea lasting about four days, 6–8 stools being passed per day. The patient complained of cramps nocturnally and swelling of his legs for the last month. Asthenia was a prominent symptom. He had not been abroad.

On examination he was thin and generalized wasting was present. Pigmentation was generalized, being most marked in the flexures and in some scars on the forearm. There was no pigmentation of the mucous membrane of the mouth and lips. There was atrophic glossitis and a distended and tympanitic abdomen. Oedema of the legs was present to just below the knees. Chvostek's and Trousseau's signs were negative. Blood pressure 90/65. The stools were pale, frothy and fatty in appearance, with a considerable bulk of fluid in addition (about 750 c.c. per stool). Up to the time of admission he had passed six or more stools daily, but on admission this diminished to about three large stools per day.

A blood count showed 4,100,000 red cells, haemoglobin 76 per cent., colour index 0·93, 6,900 white cells, with a normal differential count. Examination of the stool revealed no pus or blood, but fatty acid crystals and undigested meat fibres were present. Analysis showed 40·8 per cent. free fatty acid, with 6·1 per cent. neutral fat. A histamine test meal showed an achlorhydric resting juice, but after histamine injection there was 0·15 per cent. free hydrochloric acid.

Three days after admission the serum sodium was 291 mg. per 100 ml., and plasma proteins 4·6 per cent. This was attributed to considerable loss of base from the bowel, the low plasma protein accounting for the marked oedema of legs. Seven days later plasma chloride was 460 mg. NaCl per 100 ml,. and the urinary chloride was 0·440 g. per 100 ml. The plasma protein had risen to 6·6 per cent., with almost complete loss of the oedema. Serum calcium was 10·0 mg. per 100 ml.

Three weeks later the patient was given a low sodium chloride, high potassium diet, equivalent to 3·4 g. of sodium chloride, and 4·5 g. potassium ion daily. In addition, potassium citrate gr. 75 twice daily was given, making a total potassium intake of 8·1 g. At the commencement of the test blood pressure was 90/65, and remained steady at or about this figure during the 96 hours of the test diet. The patient felt well throughout, and even said he felt better, but passed six large stools during each of the second and third 24-hour periods.

Table I shows the urinary chloride output and blood analyses during the test.

TABLE I.

Period.	Urine volume, c.c.	NaCl. g. per 100 ml.	NaCl. g. per 24 hours.
1st 24 hours	2130	0·425	9·06
2nd ,,	1120	0·261	2·92
3rd ,,	1050	0·151	1·58
4th ,,	400	0·110	0·44

After 52 hours: Serum sodium = 290 mg. per 100 ml.
Serum potassium = 18 ,,
Plasma NaCl = 510 ,,

A week after the conclusion of this test the blood urea was 14 mg. per 100 ml. and the urea clearance 130 per cent. of maximum clearance. Noon blood sugar 70 mg. per 100 ml. ; plasma protein 5·3 per cent. ; serum sodium had risen to 316 mg. per 100 ml. and plasma chloride to 520 mg. per 100 ml. Corresponding to this the urinary chloride concentration was then 0·596 g. per 100 ml.

TABLE II.—*Values of Serum Sodium and Potassium (mg. per 100 ml.) and Plasma Chloride (mg. Cl per 100 ml.) after 52 Hours of High Potassium, Low Sodium Diet.*

Sodium.

	Addison's.		Non-Addison's.
Cutler, Power and Wilder	247–292 (*280*)		295–325 (*308*)
Willson *et al.*	272–306 (*288*)		282–335 (*310*)
Authors' case		290	

Potassium.

	Addison's.		Non-Addison's.
Cutler, Power and Wilder	18·8–29·5 (*21·5*)		14·5–26·2 (*18·4*)
Willson *et al.*	17·2–27·2 (*19·5*)		14·4–30·7 (*19·8*)
Authors' case		18·0	

Chloride.

	Addison's.		Non-Addison's.
Cutler, Power and Wilder	271–337 (*314*)		324–367 (*342*)
Willson *et al.*	290–338 (*317*)		310–367 (*343*)
Authors' case		315	

It will be seen that both before and after the period of the test diet the plasma chloride was less than 530 mg. per 100 ml. (460 and 520), whilst the urinary concentration corresponding to these figures exceeded 0·200 g. per 100 ml. (0·440 and 0·596). These figures satisfy the criteria of Anderson and Lyall (1937) for Addison's disease, diabetes mellitus and nephritis being excluded. However, if the data are compared with those of Cutler, Power and Wilder (1938), and Willson, Robinson, Power and Wilder (1942), Table II, it will be seen that with respect to the serum sodium, after 52 hours of the high potassium dietary regime, the value corresponds to the lower limit of the non-Addisonian cases and upper limit of the Addisonian.

It is also significant that the value is not lower than it was before the beginning of the test (291 mg. per 100 ml.), indicating a sodium retention during the test (cf. the retention of chloride as shown by the urinary figures). The potassium figures show such a wide range in both series of cases, and the mean values are so nearly the same, that it appears that the analysis might be discarded as a diagnostic point. The value for plasma chloride after 52 hours of the test, like that for sodium, is well within the Addisonian range, and just within the range of the non-Addisonian controls. Taking the sodium and chloride figures together, the suggestion is that they fall within the Addisonian class, rather than the reverse, but this is not conclusive.

Turning now to an analysis of the urinary output, Table III, more definite conclusions may be drawn :

TABLE III.—*Urinary Output of Chloride after 52 Hours of High Potassium, Low Sodium Chloride Intake, mg. Cl per 100 ml.*

Chloride.

	Addison's.	Non-Addison's.
Cutler, Power and Wilder . .	229–356 (*293*) .	17–141 (*54·3*)
Willson *et al.*	200–400 .	10–150
Authors' case	108	

Rate of Urine Flow, c.c. per minute.

	Addison's.	Non-Addison's.
Cutler, Power and Wilder . .	0·36–1·51 (*1·13*) .	0·52–6·2 (*3·10*)
Willson *et al.*	— .	—
Authors' case	0·78	

According to Cutler, Power and Wilder (1938), a urinary chloride concentration at the end of 52 hours in excess of 225 mg. Cl per 100 ml. indicates abnormal adrenocortical function, whilst values below 125 mg. per 100 ml. are normal. The intervening range is of doubtful significance. Willson *et al.* (1942) on the other hand give a diagram indicating that for a final plasma level of 315 mg. Cl per 100 ml., the urinary concentration should not be much in excess of 100 mg. per 100 ml. The values for the present case therefore fall within the normal range according to both sets of authors, and particularly according to the figures quoted by Cutler, Power and Wilder.

If the figures for the urinary output of chloride are considered, it will be seen that, with the exception of the period of the first 24 hours of the test, where the potassium citrate caused a considerable diuresis, there is a marked salt retention (Table IV), despite the fact that the urinary concentrations are high. The ratio of the increase of plasma sodium and chloride from the first day of the period to the 30th day is in

the ratio 1 : 1·44, whilst the atomic ratio sodium : chloride is 1 : 1·54—a good agreement.

TABLE IV.—*Chloride Retention with Various Dietary Levels of Sodium Chloride.*

Day.	"Diet," g. NaCl.	Plasma chloride, NaCl. mg. per 100 ml.	Vol. urine, c.c.	Urine, NaCl g. per 100 ml.	G. NaCl excreted per day.
1	8	460	1200	0·440	5·3
21	3·4	—	2130	0·425	9·06
22	,,	—	1120	0·261	2·92
23	,,	510	1050	0·151	1·58
24	,,	—	400	0·110	0·44
25	8	—	570	0·404	2·30
26	,,	—	1420	0·262	3·72
30	,,	520	370	0·596	2·20

It seems therefore that the criteria of Anderson and Lyall (1937), relying as they do on urinary concentration of chloride only, may be misleading, and not indicate the retention of chloride that is in fact occurring. By increasing the stringency of the conditions imposed upon the patient by the routine of Cutler, Power and Wilder (1938), an inability to retain sodium chloride is more likely to be demonstrated.

Finally, it might be mentioned that in the series of 16 cases of Addison's disease described by Willson *et al.* (1942), 10 developed symptoms of Addisonian crisis at varying periods before the completion of the 52 hours of the test, the earliest being after 8½ hours. The present case showed no symptoms after continuing the test for 96 hours—a longer period than that employed by these workers, who also indicate the dangers of the test.

On clinical grounds and from the data presented above, it was considered that there was no evidence of adreno-cortical hypofunction in the present case.

SUMMARY.

1. A case of steatorrhoea is described and analyses of blood sodium and chloride given.

2. The patient was kept on a high potassium, low sodium chloride diet, and shown to retain chloride satisfactorily.

3. The conditions of Anderson and Lyall (1937) for the urinary concentration of chloride in Addison's disease are not considered to be sufficiently stringent.

4. The findings were not considered to favour the existence of adreno-cortical insufficiency in the patient discussed.

Our thanks are due to Prof. O. L. V. de Wesselow for permission to publish the details of this case, which was under his care, and to Dr. W. J. Griffiths for advice.

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A MODIFIED LAEVULOSE (FRUCTOSE) TOLERANCE TEST COMPARED WITH THE HIPPURIC ACID SYNTHESIS TEST OF HEPATIC EFFICIENCY.

J. B. RENNIE.

From the Gardiner Institute of Medicine, University of Glasgow, and the Western Infirmary, Glasgow.

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THE literature of laevulose metabolism has been extensively reviewed by Steinitz (1937), by Stewart, Scarborough and Davidson (1938), and by Herbert and Davison (1938). From their work it appears reasonable to conclude that the liver plays at least a major part in the disposal of laevulose. The laevulose tolerance test is based on the experience that ingested laevulose is removed from the portal blood by the liver, only a small amount passing through to the systemic circulation unchanged. A defect in this mechanism is accordingly revealed by a rise in the laevulose content of the systemic blood to a level higher than is found in normal subjects similarly tested. For the procedure to be of value as a test of hepatic efficiency laevulose must be estimated. Parallel observations with laevulose and glucose curves after 50 g. of laevulose by mouth (Stewart, Scarborough and Davidson, 1938) confirmed what many have suspected, that glucose curves, after administration of laevulose, do not give reliable information as to the functional efficiency of the liver.

In the test as advocated by Stewart, Scarborough and Davidson (1938), five samples of blood were taken after fasting and at half-hourly intervals for two hours after ingestion of 50 g. of laevulose. Herbert and Davison (1938) omitted the sample at half an hour. Later Herbert (1939) estimated laevulose at the half-hour point and omitted that at one and a half hours. Four or five venipunctures are thus required. To obviate this a micro-method, using capillary blood, has been used, but in my hands it has not proved satisfactory owing to the very slight development of colour in the specimen.

In a personal communication Dr. C. P. Stewart affirmed that estimation of blood laevulose, half and one hour after ingestion of 50 g., would yield information sufficient for clinical purposes. Consideration of the figures of Stewart, Scarborough and Davidson (1938), Herbert and Davison (1938) and Herbert (1939) shows that in the great majority of cases the maximum level of blood laevulose was in fact attained within one hour of ingestion of laevulose. With regard to the exceptions the position is as follows: In controls any further rise later than one hour was insignificant. In curves signifying diminished laevulose tolerance, where blood laevulose did not reach a peak within an hour, in only one instance out of 77 observations did it fail to reach a level higher than in any of the controls. The likelihood of gaining further information is therefore too slight to justify prolonging the curve beyond one hour.

There is also justification for omission of the fasting sample. It is widely believed that there is no laevulose in fasting blood; but with diphenylamine methods a blue colour is obtained in the fasting specimen. This is attributed to oxalate by Stewart,

Scarborough and Davidson (1938) and is not taken into account. Herbert and Davison (1938) attribute the blue colour to glucose, and express their results in terms of a rise above the apparent fasting-level. Laevulose ingestion by diabetics is associated with a great increase of blood glucose but no abnormal rise in blood laevulose (Scott, 1935 ; Stewart, Scarborough and Davidson, 1938). Herbert (1938) found that a rise of blood glucose of 100 mg. per 100 ml. produced an apparent increase of blood laevulose of 2·5 mg. per 100 ml. An increase of blood glucose of that order after ingestion of laevulose never occurs, however, in those not subject to diabetes mellitus.

This review of previous work supports the opinion that the test would lose little of its value by omitting the samples obtained before ingestion of laevulose and one and a half and two hours after.

OBSERVATIONS.

This work is part of a larger investigation on hepatic efficiency, for which the laevulose tolerance test was selected to measure the part played by the liver in carbohydrate metabolism. Because four or five venipunctures were found inconvenient to the patients and to the staff, a shorter procedure, as described in this paper, was followed. In a recent paper (Rennie, 1942) the use of the hippuric acid synthesis test was described. The results obtained have been included in the tables of this paper where possible to enable a comparison to be made with the laevulose tolerance test. The two tests were usually carried out on consecutive days.

Methods.

Fifty grammes of laevulose were given to the fasting subject and samples of blood taken half and one hour afterwards. Laevulose was estimated by the method of Herbert (1939) in plasma which was found by her to contain the same amount of laevulose as the whole blood. Plasma bilirubin was estimated by the Lovibond comparator. Hippuric acid was estimated by the method of Quick (1936).

Control Cases.

The controls, 30 in number, consisted of members of the staff or of patients who were convalescent from disease not involving the liver. The results are given in Table I.* Ten of the 30 tests gave a laevulose value higher at half an hour than at one hour. The range was 5·5 to 14·0 mg. per 100 ml. The figures obtained by Herbert (from which I have not subtracted the apparent fasting levels) ranged from 6·6 to 17·0 mg. per 100 ml. in 26 patients. The frequency distribution of the two series is shown in Table I. In this paper a value of more than 15·0 mg. per 100 ml. of laevulose is considered abnormal.

The excretion of benzoic acid as hippuric acid by normal controls ranged from 2·40 to 4·39 g. in four hours (Rennie, 1942).

Hepatic Cases.

Acute hepatitis.—Cases 1–22 were examples of acute infective hepatitis (Table II). Cases 1–9 showed no diminution of laevulose tolerance. The disease was considered to be present in mild form as indicated by the slight jaundice, absence of enlargement of the liver and rapid recovery. In some patients the test was not done till improvement had well begun. Cases 10–18 were more acute and showed one or more positive tests, but all returned to normal eventually. Cases 19 and 20 represented severe

* The results in detail are omitted to save space.

attacks, and the latter still showed some diminution of tolerance when last seen three months from the onset of disease. Case 21 was a young man with an eight-months' history of moderate painless jaundice; the liver was very small. Case 22 had a moderate attack and was investigated only once, late in the disease; there was slight diminution of laevulose tolerance. In Cases 23–29 a known toxic agent was present (Table III). It is probable, however, in accordance with modern opinion, that the

TABLE I.—*Frequency Distribution of Tests on Controls.*

Plasma laevulose, mg. per 100 ml.	Number of cases.	
	Rennie.	Herbert.
5–7	3	2
7–9	5	2
9–11	8	15
11–13	12	3
13–15	2	1
15–17	0	3
	30	26

TABLE II.—*Acute Infective Hepatitis.*

Case No.	Date.	Plasma laevulose, mg. per 100 ml.		Plasma bilirubin, mg. per 100 ml.	Benzoic acid as hippuric acid, g. in 4 hours.
		½ hour.	1 hour.		
1	2.vi.41	10·4	14·5	—	1·34
2	17.vi.41	7·8	9·3	2·0	1·52
3	26.vi.41	12·6	11·7	2·0	2·03
4	21.ii.42	10·9	7·1	5·8	2·58
5	28.v.42	9·3	7·6	6·0	3·12
6	31.viii.42	8·4	8·3	3·8	1·69
7	7.xii.42	11·5	9·0	0·7	3·19
8	22.i.42	10·3	12·3	2·0	2·47
9	3.iv.42	14·3	12·5	8·8	1·16
	16.iv.42	11·8	14·8	—	2·06
	24.iv.42	7·8	9·5	3·0	2·82
10	3.x.41	22·2	20·8	16·2	0·86
	17.x.41	10·5	18·2	3·0	1·75
	25.x.41	13·0	13·8	1·6	2·62
11	7.x.41	27·4	45·4	20·0	1·06
	28.x.41	16·8	24·1	3·0	2·20
	3.xi.41	20·6	13·8	1·8	2·48
	24.xi.41	15·0	13·9	1·3	2·43
12	17.xi.41	26·3	23·8	22·0	0·89
	24.xi.41	13·8	12·2	4·4	1·86
	1.xii.41	18·2	22·2	2·4	1·06
	8.xii.41	12·9	11·8	1·0	2·31
13	1.xii.41	20·0	16·6	9·0	2·46
	8.xii.41	7·1	6·1	0·7	2·22
14	7.iv.42	15·1	26·6	35·0	0·44
	14.iv.42	15·5	19·0	26·0	0·65
	21.iv.42	12·1	15·0	7·5	1·11
	28.iv.42	13·2	—	3·0	0·60
	4.v.42	12·8	12·2	3·0	0·74
	12·5·42	11·1	8·1	1·4	0·92
	27.vii.42	9·3	9·8	0·2	1·20
15	15.ix.42	18·3	25·0	16·0	0·47
	22.ix.42	19·2	26·3	12·5	0·69
	29.ix.42	14·1	14·9	6·5	1·20
	6.x.42	12·5	11·6	4·5	2·38
16	17.xi.42	15·1	20·8	12·0	0·86
	24.xi.42	22·7	22·5	12·0	—
	7.xii.42	11·7	13·2	5·0	1·34
	24.xii.42	—	—	—	3·71
17	18.xii.42	19·0	15·8	7·0	1·57
	28.xii.42	7·8	13·9	1·2	1·35
18	10.xii.42	12·0	15·4	1·7	2·79
	16.xii.42	7·1	7·1	0·7	—
19	1.vii.42	23·5	30·3	15·0	0·68
	7.vii.42	36·4	26·6	9·0	0·53
	15.vii.42	18·1	17·7	2·7	0·83
	22.vii.42	19·4	22·2	1·8	0·79
	29.vii.42	20·4	20·2	1·8	1·39
	7.viii.42	22·3	20·9	0·6	1·56
	13.viii.42	24·4	16·2	0·6	0·92
	19.viii.42	12·5	17·1	0·4	1·75
	26.viii.42	19·0	16·4	0·5	1·84
	28.ix.42	12·5	10·4	0·2	2·01
20	10.vii.42	25·6	15·1	20·0	0·16
	15.vii.42	35·1	26·6	20·0	0·28
	22.vii.42	26·6	34·4	18·0	0·64
	29.vii.42	36·4	30·5	18·0	0·84
	7.viii.42	25·0	26·6	10·0	0·98
	13.viii.42	24·4	16·2	3·0	0·92
	19.viii.42	19·3	10·9	2·6	1·45
	28.viii.42	19·2	17·5	1·8	1·72
21	27.vi.41	26·3	34·0	12·0	0·60
22	16.xii.42	16·6	14·4	1·0	3·66

TABLE III.—*Acute Toxic Hepatitis.*

Case No.	Date.	Plasma laevulose, mg. per 100 ml. ½ hour.	Plasma laevulose, mg. per 100 ml. 1 hour.	Plasma bilirubin, mg. per 100 ml.	Benzoic acid as hippuric acid, g. in 4 hours.	Remarks.
23	2.ix.41	27·0	31·7	4·8	0·47	Syphilis and arsenic.
24	17.xi.42	35·7	29·8	13·0	0·51	„ „
	23.xi.42	35·1	31·7	7·0	1·04	
	1.xii.42	7·2	8·3	3·6	2·08	
25	29.x.42	5·0	8·5	5·4	1·15	„ „
26	10.xii.42	15·1	14·3	3·3	—	„ „
27	20.xii.42	17·5	20·2	1·8	—	„ „
28	25.iv.42	12·4	16·3	10·0	1·63	Arthritis and gold.
	4.v.42	8·2	10·7	5·5	1·41	
	19.v.42	13·4	12·4	1·3	1·68	
29	26.v.42	16·2	21·5	6·8	0·91	Burns.
	5.vi.42	9·4	10·7	0·9	3·63	

TABLE IV.—*Secondary Carcinoma of the Liver.*

Case No.	Date.	Plasma laevulose, mg. per 100 ml. ½ hour.	Plasma laevulose, mg. per 100 ml. 1 hour.	Plasma bilirubin, mg. per 100 ml.	Benzoic acid as hippuric acid, g. in 4 hours.
30	30.v.41	8·8	7·6	<0·2	1·18
31	14.vii.41	35·7	22·7	<0·2	1·50
32	13.ix.41	10·5	8·7	<0·2	0·31
	1.xi.41	8·0	11·1	0·3	—
33	8.xii.41	15·1	14·4	1·0	1·50
34	20.ii.42	6·7	8·8	2·0	2·32
35	24.ii.42	3·5	9·7	1·4	1·41
36	26.ii.42	16·0	21·5	2·7	1·07
37	26.ii.42	6·5	6·7	8·0	0·38
38	3.iii.42	7·6	8·9	<0·2	1·35
39	10.iii.42	11·1	10·0	<0·2	2·18
40	19.vi.42	7·4	11·1	0·2	1·39
41	10.viii.42	11·1	9·9	<0·2	1·99
42	8.viii.42	20·4	19·0	1·0	0·36
43	27.xi.42	16·6	17·8	1·0	2·16
44	11.xii.42	10·2	9·7	1·2	1·69
45	6.i.43	14·0	11·5	5·0	0·42

TABLE V.—*Obstructive Jaundice.*

Case No.	Date.	Plasma laevulose, mg. per 100 ml. ½ hour.	Plasma laevulose, mg. per 100 ml. 1 hour.	Plasma bilirubin, mg. per 100 ml.	Benzoic acid as hippuric acid, g. in 4 hours.	Remarks.
46	25.vi.41	9·9	11·3	18·0	2·62	Ca. head of pancreas.
47	7.vii.41	9·0	10·0	17·0	2·05	„ „
	13.xi.41	10·4	11·9	<0·2	1·36	After cholecyst-enterostomy. Liver now invaded.
48	29.xi.41	8·0	10·9	10·5	0·86	Ca. head of pancreas.
49	6.x.42	18·5	23·0	14·0	1·04	„ „
50	29.viii.42	12·0	17·5	22·0	0·38	„ „
51	31.x.41	6·7	7·1	16·0	0·33	„ „
	25.xi.41	12·0	13·3	21·1	1·03	After intensive glucose therapy.
52	27.iii.42	14·8	16·6	11·5	0·96	Ca. head of pancreas.
	2.iv.42	5·1	6·6	17·0	1·13	
	7.iv.42	8·7	6·9	36·0	0·78	
	13.iv.42	10·8	12·4	28·0	0·79	After intensive glucose therapy.
53	24.v.42	5·5	7·0	12·5	0·92	Ca. head of pancreas. Diabetes.
	3.vi.42	15·4	15·9	13·0	0·50	After intensive glucose therapy.
54	27.viii.41	16·3	25·4	16·0	0·33	Ca. head of pancreas.
	15.x.41	7·0	11·4	—	1·56	8 days after cholecyst-jejunostomy.
	27.x.41	9·1	12·2	0·7	2·08	

toxic agent merely rendered the liver more liable to attack by the virus of infective hepatitis. Six of these seven patients showed diminished tolerance.

Secondary carcinoma of the liver.—Sixteen patients were investigated (Table IV). All had an advanced degree of hepatic involvement, secondary to neoplasm of stomach or bowel. In all but one (Case 37) the liver was very greatly enlarged. Laevulose tolerance was diminished in five, in two of these only slightly.

TABLE VI.—*Cirrhosis of the Liver.*

Case No.	Date.	Plasma laevulose, mg. per 100 ml. ½ hour.	Plasma laevulose, mg. per 100 ml. 1 hour.	Plasma bilirubin, mg. per 100 ml.	Benzoic acid as hippuric acid, g. in 4 hours.	Remarks.
55	12.ii.42	21·0	19·4	1·3	1·23	Jaundice three years before.
56	30.vi.41	15·6	11·8	1·2	2·08	Jaundice two years before.
57	12.ii.42	26·6	33·3	1·2	1·23	Cause unknown.
58	3.vii.41	26·6	40·0	0·2	0·94	Jaundice three years before.
	11.viii.41	23·5	28·7	3·0	1·21	
59	17.v.41	7·4	8·2	—	1·37	Early haemochromatosis.
60	25.vi.41	15·1	16·1	12·0	1·58	Cause unknown.
61	27.iii.42	11·9	20·0	0·6	1·92	Jaundice nine months before.
62	5.iv.42	15·8	19·1	1·8	1·06	Cause unknown.
	16.iv.42	14·8	18·8	0·6	1·01	
63	12.ii.41	31·0	45·0	—	—	,, ,,
64	16.xii.42	26·3	19·2	5·0	1·53	,, ,,
	7.i.43	18·4	32·5	8·0	1·50	
65	20.x.42	25·6	31·2	2·5	0·44	,, ,,
66	17.xi.41	15·4	19·6	1·2	2·89	Splenic anaemia.
67	29.i.42	20·0	24·0	0·9	1·71	,, ,,
68	7.ii.42	12·3	18·0	<0·2	1·96	,, ,,
69	2.iv.42	16·1	13·0	0·4	1·09	,, ,,
70	28.v.42	22·7	19·4	2·6	1·52	Biliary cirrhosis. Traumatic stricture of left hepatic duct. Biopsy.

TABLE VII.—*Disease of the Biliary Tract.*

Case No.	Date.	Plasma laevulose, mg. per 100 ml. ½ hour.	Plasma laevulose, mg. per 100 ml. 1 hour.	Plasma bilirubin, mg. per 100 ml.	Benzoic acid as hippuric acid, g. in 4 hours.	Remarks.
71	12.v.42	15·4	10·5	6·0	0·59	Stones in G.B. and C.D. Cholangitis.
	19.v.42	11·5	9·3	2·4	1·03	After intensive glucose therapy.
	28.v.42	11·1	11·9	2·4	0·83	,, ,, ,,
72	31.vii.42	14·7	14·0	15·0	0·79	Stones in G.B. and C.D. Cholangitis.
	12.viii.42	15·4	18·5	3·0	0·37	After intensive glucose therapy.
73	30.ix.42	11·6	12·0	10·5	2·03	Stones in G.B. and C.D.
	8.x.42	14·9	13·1	7·0	1·99	
74	23.vii.42	9·7	12·0	7·5	2·69	,, ,,
75	11.xii.42	7·4	8·1	3·6	3·21	,, ,,
76	19.v.42	7·1	7·8	4·0	2·72	Stone in C.D.; G.B. removed two years ago.
77	1.v.41	7·8	8·2	—	2·48	Cholecystitis.
78	4.ix.41	8·0	9·3	<0·2	2·96	,,
79	27.xii.41	10·2	10·2	0·3	3·97	,,
80	2.vi.42	13·4	12·0	<0·2	3·00	Stones in G.B.
81	2.iv.42	9·2	11·5	<0·2	2·52	,, ,,
82	7.xi.42	10·9	12·4	0·3	3·14	,, ,,
83	12.vii.42	8·8	7·7	0·2	2·05	,, ,,
84	9.viii.42	11·1	9·9	0·4	2·72	,, ,,
85	6.vi.42	11·0	10·0	<0·2	2·46	,, ,,
86	11.viii.42	8·0	7·2	<0·2	2·49	,, ,,
87	10.vii.42	10·8	9·0	0·2	3·32	,, ,,

Obstructive jaundice.—Of the nine cases in this group (Table V), five showed diminution of laevulose tolerance, but only in Cases 49 and 54 was the rise in plasma laevulose a marked one. Improvement in tolerance appeared to follow relief of obstruction in Case 54.

Cirrhosis of the liver.—Laevulose tolerance was diminished in 15 out of 16 patients (Table VI).

Disease of the biliary tract.—In this group of 17 cases (Table VII) all showed normal laevulose tolerance except Cases 71 and 72, who suffered from severe cholangitis.

A summary of the results in 87 patients is given in Table VIII. The laevulose tolerance and hippuric acid synthesis tests were carried out at approximately the same time in 84 patients. The results are compared in Table IX.

TABLE VIII.—*Summary of Results of Laevulose Toierance Tests.*

	Number of cases.	Number with diminished laevulose tolerance.
Acute hepatitis	29	19
Secondary carcinoma of the liver	16	5
Obstructive jaundice	9	5
Cirrhosis of the liver	16	15
Disease of the biliary tract	17	2
Total	87	46
Total excluding disease of the biliary tract	70	44

TABLE IX.—*The Laevulose Tolerance Test Compared with the Hippuric Acid Synthesis Test (Initial Observations on Each Case).*

	Number of cases.	Laevulose tolerance diminished.	Hippuric acid synthesis defective.
Acute hepatitis	27	17	20
Secondary carcinoma of liver	16	5	16
Obstructive jaundice	9	4	8
Cirrhosis of liver	15	14	14
Disease of biliary tract	17	1	5
Total	84	41	63

SUMMARY AND CONCLUSIONS.

1. Plasma laevulose has been estimated on 139 occasions half and one hour after ingestion of 50 g. of laevulose by 87 patients with hepatic disease.

2. The shortened form of laevulose tolerance test gave positive results in 44 out of 70 patients suffering from acute hepatitis, secondary carcinoma of the liver, obstructive jaundice and cirrhosis. This finding is in close agreement with the work of Herbert and Davison (1938) and Herbert (1939), but shows a smaller proportion of positive results than the series of Stewart, Scarborough and Davidson (1939). Clearly not much reliance can be placed on comparisons of relatively small numbers of tests on such heterogeneous groups.

3. Positive results were most often obtained in cirrhosis and next, but considerably less frequently, in acute hepatitis. It seemed likely that more positive results would have been obtained had it been feasible to perform all the tests at an early stage in acute hepatitis. The highest values for plasma laevulose were in these two conditions.

4. The test was negative in a high proportion of cases of secondary carcinoma of the liver, although infiltration was widespread, as was proved in several cases at autopsy. Presumably the remaining liver cells were functioning adequately enough to deal with the laevulose.

5. In obstructive jaundice, even when of a long duration, the test was often negative and high values for plasma laevulose were uncommon.

6. As was to be expected the presence of biliary disease alone, with or without gall-stones, did not lower the laevulose tolerance. It was surprising that plasma laevulose was only slightly above normal in two women with cholangitis. At operation even the finer hepatic ducts dripped pus when a biopsy was made, and one of the patients died of hepatic failure two weeks after operation.

7. From earlier work and from the results given in this paper, it would appear that estimation of blood laevulose in two samples of blood, taken after 50 g. of laevulose by mouth, detects hepatic damage about as frequently as did the earlier test entailing four or five venipunctures.

8. A comparison of a modified laevulose tolerance test with the hippuric acid synthesis test was made in 84 patients. In secondary carcinoma of the liver, obstructive jaundice and disease of the biliary tract, synthesis of hippuric acid was greatly superior. In cirrhosis there was nothing to choose between the tests. In acute hepatitis the laevulose tolerance test was positive almost as often as the hippuric acid synthesis test. Repeated observations showed, however, that the results of the laevulose tolerance test tended to return to normal sooner than those of the hippuric acid synthesis test.

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OBSERVATIONS ON THE AGGLUTINATION OF FOWL RED CELLS BY INFLUENZA VIRUSES.

C. H. STUART-HARRIS.

From a Military Laboratory.

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In 1941 Hirst described the phenomenon of the agglutination of fowl red cells by preparations of influenza viruses. He infected chick embryos with influenza virus by direct inoculation into the allantoic sac, and showed that after incubation the egg fluid contained the agglutinating principle in abundance. He also showed that in the presence of a serum containing neutralizing antibodies to influenza virus as determined by mouse test, the agglutination of red cells was inhibited and that such inhibition was specific for the virus concerned—either influenza A or B. Hirst later reported (1942) that normal animal sera would also inhibit the agglutination of red cells, but in much lower dilution than sera with neutralizing antibodies for the virus. A relationship was established between the neutralization of virus by a serum as determined by mouse test and the *in vitro* agglutination-inhibition titre; the rise of influenza virus antibody in human serum after an attack of influenza was as readily demonstrated *in vitro* as by mouse test.

Preliminary Studies.

Using the amniotic and allantoic fluids from chick embryos inoculated amniotically by the technique of Burnet (1940), Hirst's observations were easily confirmed. The agglutination was as readily demonstrated on a slide or in a Petri dish with blood from the infected chick embryo as in a test-tube. Hirst's technique for measuring the inhibitory action of serum was not at first available, and in preliminary studies egg fluids and red cell suspensions were mixed in wide round-bottomed tubes and observed at intervals. When fluids were diluted serially in order to determine the titre of agglutinin, the end-point of agglutination was difficult to locate by observation at room temperature. Sedimentation for two hours at 4° C. however gave a deposit of red cells in the form of a central circular disc in the absence of agglutination, and of a plaque extending over the whole bottom of the tube when agglutination had occurred. By this technique virus fluids were titrated against serial dilutions of sera. Sera from normal rabbits, ferrets, fowl and horses were all found to possess inhibitory powers, but sera containing neutralizing antibodies to influenza virus A or B as determined by mouse inoculation were inhibitory in much higher dilutions to the homologous than to the heterologous virus. Control tests of serum and red cells without virus also showed agglutination, but only in strong concentrations of serum. Such agglutination was less obvious at room temperature, but it was sufficient to confuse the issue when the inhibitory action of the serum against the virus was weak. Thus when pairs of sera from cases of influenza collected during the febrile stage and in convalescence were examined against a fixed quantity of virus it was possible to measure the inhibitory substance in the convalescent serum, but no titre could be

assigned to the acute sample owing to the confusing effect of serum-agglutination. It was thus impossible to measure the actual rise in inhibitory antibodies and the ice-box technique was therefore abandoned. Hirst's technique for measuring the inhibitory action of serum on the virus-red cell agglutination was described in 1942. In this technique Hirst measured the intensity of agglutination by examining the supernatant fluid after one hour's sedimentation in narrow tubes (7 cm. × 0·8 cm.) at room temperature. A fixed degree of agglutination (two-plus), namely, that which lowered the red-cell content of the supernatant by more than 25 per cent. but less than 50 per cent. of the original red suspension, was taken as the end-point of the reaction. This end-point was taken both for the virus titration and the serum inhibition, and in the latter test four times the amount of virus fluid needed to produce two-plus agglutination of cells in the absence of serum was added to each dilution of serum. The titre of a serum was expressed as the reciprocal of that final dilution of serum which produced two-plus agglutination in the presence of the stated concentration of virus. Hirst did not refer to agglutination of red cells by serum alone, and no serum has been encountered in the work to be described which caused a sufficient intensity of agglutination to interfere with the inhibition test as thus performed. Hirst's technique was followed exactly in the series of tests described below, except that in the interests of economy half quantities of reagents (virus, serum and red cells) were used throughout the test. The reduction in height of the column of supernatant fluid which was thus caused made the reading of the tests slightly more difficult but did not affect the end-point. Daylight illumination was employed throughout.

Factors Producing Variation in the Inhibition Test.

Before the significance of changes in the titre of agglutinin-inhibiting substance in any individual's serum can be assessed, the variation in the titre imposed by the limitations of the test must be ascertained. In the course of the performance of the serum tests described below many instances occurred where a serum was tested on more than one occasion, and since then deliberate repetition has been employed in an attempt to discern the variables of the reaction.

1. *The Virus.*

It was impossible to use the same virus fluids throughout the series of tests. PR8 and Lee viruses in the form of mouse lung material had been obtained originally from the National Institute for Medical Research, Hampstead, propagated in mice for some passages and then adapted to eggs by amniotic passage from mouse lung filtrates. Several egg passages were necessary before fluids with good agglutinating titres were obtained, and the usual practice was to inoculate six embryos, harvest the fluids after 48 to 72 hours, pool the fluids from each batch and then use the pooled fluid until exhausted, so long as a titre of 1 : 64 or more was obtained. Before carrying out the serum-inhibition test, the virus fluid was freshly titrated, and a standard strength of fluid four times the minimal quantity needed to produce two-plus agglutination was added to each tube of the serum titration. In this way a constant amount of agglutinating agent should have been used in each test, and thus the results with different virus fluids should have been comparable. In fact, however, repeat tests of 27 sera with the same strain of PR8 virus showed a considerable variation in results. Table I shows the extent of the variation, which was much greater when different fluids were employed than when the same egg fluid was used each time (Table I). Some of these repetitions were done on a single pool of normal human sera, which was titrated each time a batch of unknown sera were tested, and the extreme titres in 18 tests with this

serum with ten different fluids were 128 and 32. Both these values were obtained twice; titres of 64 were obtained 8 times, and of 48, 6 times. Although high or low readings were obtained sometimes in an entirely unpredictable manner, a trend was observed with this serum over the course of seven months, namely, that the later values were lower on the whole than those obtained at first. This trend was noticed with other sera, and was shown clearly when the results of testing ten different sera in November, 1941, were compared with the results on the same sera in June, 1942. Nine of the sera gave lower readings in the later test, the 10th serum alone having a higher titre on retest, and this serum was the strongest of the whole batch, and was in fact a pool of human sera from convalescent cases of influenza A infection in 1941. The extent in fall of titre of the other nine sera was from two to five-fold in the different instances. It seemed probable that apart from some natural loss of titre in these sera, which were stored in the liquid state in a refrigerator during the entire period, variability of the virus was responsible for this systematic trend. The virus fluid used in the earlier tests was from the 12th generation of eggs, that in the later tests from the 21st–22nd generation. In order to confirm or refute this hypothesis, the PR8 mouse strain was readapted to eggs and tested after four amniotic passages. The Hampstead strain of egg-adapted PR8 virus obtained from Miss K. Mills of the National Institute for Medical Research was also tested after four passes in all. Both these virus fluids gave similar readings, and readings which were higher than those obtained with the PR8 egg strain after 22 passages (Table II). The greatest differences were obtained with normal sera, and in contrast the serum from a convalescent PR8 ferret gave identical values. These results appeared to confirm the theory that egg passage modified the facility with which agglutination was inhibited by serum, at any rate with this particular strain of virus.

Meanwhile tests were also carried out with yet another PR8 egg-adapted strain of virus. This was obtained in the form of dried egg fluid from Dr. G. K. Hirst, of the

TABLE I.—*Variation of Repeat Tests with PR8 Virus.*

	Same titre.	±50%.	±100%.	±200%.	±300%.	Total tests.
Same egg fluid	12	9	7	0	0	28
Different egg fluids	6	11	9	5	4	35

TABLE II.—*Comparison of Different Egg Passage Fluids of Same PR8 Strain.*

	Hampstead (4 egg passes).	Tidworth (5 egg passes).	Tidworth (22 egg passes).
Individual sera :			
Hood	40 : 48 : 56*	..	24 : 32 : 32 : 32
Weston	..	8	<8
Normal ferret	..	32	8
Convalescent PR8 ferret	..	960	960 : 960 : 960
Pooled human sera :			
Pre-vaccination	56 : 64 : 96	56 : 64	32 : 16
Post-vaccination 1	72 : 96 : 112	64 : 96	56 : 32
,, 2	48 : 64 : 80	40 : 64	64 : 80
Pool 3	40 : 40 : 56	40 : 48	24 : 24 : 32
,, 5	..	40 : 48	32 : 32
,, 2	..	24	12 : 16 : 16

* Figure indicates the ++ titre obtained in any particular test.

International Health Division of the Rockefeller Foundation, by courtesy of Dr. C. H. Andrewes, of the National Institute for Medical Research, Hampstead. The American egg virus had undergone twelve passages when received, and a further seven passages by amniotic inoculation were then carried out. Reconstituted egg fluid and pooled allantoic and amniotic fluids from eggs inoculated with this fluid and passages from it were tested in parallel with our egg-adapted virus which had been passed 22 times. Table III illustrates the wide variation in results obtained. The divergence was particularly great with individual human and normal ferret sera. More comparable values with the two viruses were obtained with the strong sera from convalescent ferrets, a hyperimmune rabbit serum and a pool of serum from convalescent human influenza A patients which was the only available human convalescent serum. Repeat tests with the U.S. egg fluids after passage did not reveal a systematic tendency for variation within the limited range of seven passes. Thus desiccation of the American virus did not explain the differences between the two virus fluids, which was maintained even with fresh fluids of the American strain. The two egg-adapted viruses were titrated by intranasal inoculation of mice, and comparable mouse titres were obtained both with egg material and after several mouse lung passages. The American PR8 virus had undergone 98 ferret and 335 mouse passages prior to its adaptation to eggs. The British PR8 virus had been obtained originally from Dr. T. Francis of the Rockefeller Foundation in 1935. Prior to its adaptation to eggs in 1940 it had undergone a further large number of mouse passages. Some virus dried at Hampstead in 1937 was obtained from Dr. C. H. Andrewes, and after a further series of 10 mouse passages it was adapted to eggs by amniotic inoculation of a mouse lung filtrate. Egg fluids after three and four passages of this virus gave serum inhibition titres closely similar to those obtained with the American strain, and higher than with any other egg fluids of the British virus. Thus the British virus adapted to eggs after a certain number of mouse passages produced egg fluids which gave higher serum inhibition titres than the same strain of virus after many more mouse passages. Still lower values were given with fluids from the latter strain after 22 amniotic egg passages. In view of the fact that all three strains gave more closely comparable values with immune serum from convalescent ferrets and humans than with normal serum, antigenic variation produced by passage did not seem to be a likely cause of the variable results. No explanation of the observed facts can yet be given, but they are recorded in order to emphasize the difficulties in determining the exact inhibition titre of any particular serum against a particular strain of influenza virus.

2. *The Red Cell.*

Hirst (1942) was unable to find differences between serum inhibition titres when red cells from different fowls were used in tests with the same virus and serum. The repeat tests described above were results with different fowl cells and influenza virus A. No significant differences which could be attributed to the cells were encountered. A few tests were, however, made with reconstituted fluid infected with influenza B virus (Lee strain) and received in dried form from Dr. G. K. Hirst. Striking differences were obtained in a test employing red cells from two fowls of the Rhode Island Red breed. The cells were prepared in the usual way as described by Hirst (1942). Only slightly different titration values for the egg fluid were obtained with the two lots of cells. Yet widely different serum inhibition titres were observed with two pools of human serum tested with the different red cell suspensions (Table IV). It appeared that the agglutination by the virus of red cells of fowl N.N. was less readily inhibited by serum than that of the cells of B.C. Analysis of many tests which had been carried

TABLE IV.—*Comparison of Different Fowl Cells and Influenza Virus B.*

		B. virus fluid titration.							
		1/8.	1/16.	1/32.	1/64.	1/128.	1/256.	1/512.	++titre.
Rhode cells	N.N.	++++	++++	++++	+++	++±	+	0	160
„ „	B.C.	++++	++++	+++	+++	+	±	0	96

Cells.	Serum.	Virus fluid dilution.	Final dilution of serum. 1/8.	1/16.	1/32.	1/64.	1/128.	1/256.	++ titre.
Rhode cells N.N.	Pool 4	1 : 10	±	+±	++±	+++	+++±	++++	24
„ „ B.C.	„ 4	1 : 6	0	0	+	+±	++±	+++	96
„ „ N.N.	„ 6	1 : 10	+±	++±	+++	+++±	++++	++++	12
„ „ B.C.	„ 6	1 : 6	0	0	+	+±	++±	+++	96

out using our own strain of Lee virus showed that often when fowl B.C.'s blood had been employed the entire readings of the serum inhibition titres were higher than when blood from other Rhode Island Red fowls had been used. Comparison of cells from two Brown Leghorn fowls and a Light Barred Cross with the same virus fluid and sera showed no differences. All gave readings agreeing with B.C.'s blood. A single test with blood from a fowl of a Rhodes-Light Sussex Cross gave values intermediate between the high serum titres of B.C. and the low ones of N.N. After some months the tests with B.C. and N.N. were repeated using the same dried B fluid and four different sera. Smaller differences were now obtained, although the readings with B.C. blood were still uniformly higher. However, a second comparison of N.N. red cells with blood from two Brown Leghorn fowls showed the same striking differences as before. The serum titres with N.N. blood were 1/3 to 1/6 as high as with the Brown Leghorn cells. It thus appeared that systematic differences in serum inhibition titres with influenza virus B might be encountered when different fowls' red cells were employed, and even when the same fowl's red cells were used at different seasons of the year. The effect was the same in each case, namely, that the serum was apparently less able to inhibit the agglutination of some red cell suspensions than of others. It was found to be present not only with a pooled serum from normal humans, but also with a hyperimmune influenza B rabbit serum prepared by inoculating a rabbit with mouse lung suspensions intraperitoneally. A small series of tests with red cell suspensions which gave different serum inhibition titres against virus B were made with virus A, but no similar systematic differences were found.

Examination of Pairs of Human Sera.

It was shown above that so long as the same virus fluid and the same red cell suspension was employed, the observed inhibition titre of any particular serum with influenza virus A varied within the limits of ± 100 per cent. Many tests were carried out with pairs of human sera collected from cases of acute respiratory infection during 1941 and 1942. In each case both sera from any particular individual were always tested against the same virus fluid with the same virus suspension on the same day. A pool of normal human serum was examined at the same time as a batch of unknown pairs of sera were tested against virus A ; a serum from a rabbit immunized with mouse lung suspension of influenza virus B was similarly used in tests of sera against virus B. The following pairs of sera were examined in small batches :

(1) "Acute" and "Convalescent" pools of serum from cases of influenza A in 1941 as proved by mouse neutralization tests (Stuart-Harris, 1941). Sera from individual cases which had been tested in the preliminary studies described above were pooled prior to the adoption of the Hirst technique and so were not available for later test in their original form. Nevertheless the two pools showed wide differences in

TABLE III.—*Comparison of Agglutinin Inhibition Titres of Various Sera with Two Egg-adapted PR8 Virus Strains.*

	Single human sera.						Pooled human sera.						Normal ferrets.			Immune sera.			
	Salter.	Weston.	Hood.	Hicks.	Dance.	Nielson.	Pool 3.	Pool 2.	Pool 1.	Pool 7.	Pool 5.	Pool 8.	66.	58.	Pool.	Convalescent human.	PR8 ferret.	PR8 ferret.	PR8 rabbit.
American PR 8 virus	128	128	128	96	64	48	192	128	128	192	96	80	256	32	32	512	800	1920	320
	..	..	64	..	..	..	192	96	96	64	64	56	128	..	24	..	640	1280	240
	..	..	64	..	..	..	128	64	96	..	48	..	..	..	..	..	640	1600	240
British PR8 virus	24	12	32	48	<8	64	32	24	64	96	32	32	48	<8	<8	384	1280	1920	160
	..	8	24	..	..	..	24	16	48	64	32	24	..	..	..	..	960	960	120
	..	8	16	..	..	..	24	16	48	64	32	24	..	..	..	..	960	1120	..

* Different figures in the same column record the various results obtained with the same or different virus fluid on different occasions.

TABLE V.—*Influenza A—Serum Inhibition Tests.*

Sera.	Final dilution of serum. 1/8.	1/16.	1/32.	1/64.	1/128.	1/256.	1/512.	1/1024.	Titre.
Normal rabbit	++	++	+++	+++	++++	++++	−	..	16
Convalescent PR8 ferret	0	0	0	0	0	+	++	+++	512
Influenza "A" Pool acute 1941	+	++	+++	++++	++++	++++	−	..	16
Influenza "A" pool convalescent 1941	0	0	0	0	±	+	+++	++++	384
Clinical influenza 1942 acute Case 1	0	+	++	+++	+++	+++	−	−	32
„ „ „ convalescent „	0	+	++	+++	+++	+++	−	−	32
„ „ „ acute Case 2	+	++	+++	+++	+++	++++	−	−	16
„ „ „ convalescent „	±	+	++	+++	+++	++++	−	−	32

inhibition titre to virus A (Table V, p. 38), whereas both sera had the same inhibition titre to virus B. The convalescent pool thus possessed 24 times the inhibitory power to virus A of the acute serum, which was a difference many times the variation to be expected from technical causes.

(2) Eighteen pairs of sera from individuals vaccinated with a chick embryo vaccine of influenza virus A (Horsfall, Lennette and Rickard, 1941) collected before and 14 days after vaccination. These sera, which were only tested against virus A, showed identical titres in five instances. Three post-vaccination sera showed increments of 50 per cent. and one a decrease in titre of 50 per cent. compared with the pre-vaccination samples. Seven post-vaccination sera had titres 100 per cent. and two had titres 300 per cent. in excess of the first sample. While an increase in titre of 9 out of 18 sera of the order of 100 per cent. or more was probably significant, only those sera with 4-fold increases of inhibition titre were considered to have been significantly altered. Many of these sera were also tested by Miss D. Lush at the National Institute for Medical Research both *in vitro* by Hirst's technique and by mouse inoculation. Good correlation was obtained between inhibition and neutralization titres in the various instances.

(3) Fifty-two pairs of sera from sporadic cases of acute respiratory infection collected during the winter of 1941–42, when influenza did not occur as a localized or a spreading epidemic. Each pair comprised an acute and convalescent serum from each patient. Thirty-four pairs were from cases of clinical influenza and 12 pairs were from cases of " pneumonitis " or atypical pneumonia, 6 being from the Canadian Army and sent for test by courtesy of Major N. E. Mackinnon, R.C.A.M.C., and 6 from a single British unit in March, 1942. All of the sera were tested against both virus A and B. In no case did a pair of sera exhibit differences in inhibition titre to either virus greater than 100 per cent. Examples of the results with two such pairs are given in Table V. Thirty pairs of sera gave identical titres against virus A, 39 against virus B. Thirteen convalescent sera had titres 50–100 per cent. in excess of the acute level against virus A, and 7 had similar increments against virus B. Decrease in titre of between 50–100 per cent. occurred in 9 sera with virus A and 6 with virus B. There was no observed correlation between change or lack of change in titre against one virus compared with that exhibited to the other virus. In view of the variations in titre of any particular serum tested under different circumstances as described above, the relative constancy in the results with these pairs of sera was remarkable. It was probably attributable to the fact already emphasized that the two members of a pair of sera were always tested against the same fluid and red cell suspension. No pair of sera among the 52 individuals studied thus showed serological differences of the degree encountered in the pooled sera from 1941 when influenza A was prevalent (Andrewes, Glover, Hudson, Lush and Stuart-Harris, 1941). In order to control these findings, 6 pairs of 1942 sera, 4 from typical cases of clinical influenza and 2 from cases of pneumonitis, were tested in mice for neutralizing antibodies to virus A and B. No pair of sera exhibited significant change in titre of virus-neutralizing antibody. Attempts were also made to recover virus from garglings of patients by the procedure which had been successful in 1941 (Stuart-Harris 1941). Five garglings were filtered and inoculated into chick embryos by the amniotic route. Passage from chick to chick was carried out for at least three passages and the egg fluids were repeatedly examined for the presence of red-cell agglutinins. Two different samples of chick material were inoculated into ferrets by Mr. R. E. Glover, of the Mill Hill Laboratory of the National Institute for Medical research. No virus was recovered by these procedures. Further, two individual garglings and a pool of four others were inoculated by Mr. Glover into ferrets. No reaction of any sort was

observed in these ferrets, which were not immune to later tests with W.S. virus. Thus the absence of change in inhibitory substance in representative 1942 sera was correlated with an absence of change in neutralizing antibody to influenza virus and a failure to recover virus from the individual patients.

SUMMARY.

1. Study of the phenomenon of agglutination of fowl red cells by influenza viruses, and inhibition of the agglutination by serum, indicates that the technique described by Hirst (1942) is preferable to titration methods which determine the end-point in terms of the presence or absence of agglutination.

2. Factors causing variation in the inhibition titre of any particular serum are described. Different egg fluids infected with the same strain of virus and different strains of the same PR8 virus gave greater differences in serum inhibition titres than did the same egg fluid when examined repeatedly. Red cell suspensions from different fowls gave different serum inhibition titres with virus B, but not with virus A.

3. Pairs of human sera from cases of acute respiratory infection in the winter of 1941–42 did not exhibit significant differences in inhibition titres to either virus A or B. Mouse neutralization tests and attempts to recover virus from garglings also failed to reveal evidence of influenza virus infection in a limited number of these cases. These results contrasted with those obtained with material from cases of influenza in the winter of 1940–41, which yielded evidence of virus A infection by all three methods.

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